

Functional imaging of neural systems associated with alcohol reward and amphetamine toxicity

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The cover layout design by Mateusz Dudek. The images were acquired and processed by Mateusz Dudek and show rat brain activation after chronic alcohol exposure.

“...If when you say whisky, you mean the devil’s brew, the poison scourge; the bloody monster that defiles innocence, yea, literally takes the bread from the mouths of little children, then certainly I am against it with all my power...But, if when you say whisky, you mean the oil of conversation; the stimulant drink that puts the spring in the old gentlemen’s step on a frosty morning; the drink that enables a man to magnify his joy, and his happiness, then certainly I am in favor of it. This is my stand. I will not retreat from it. I will not compromise.”

Mississippi State Senator, Judge Noah S. Sweat, addressing the various aspects of the impact of alcohol on society

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LIST OF PUBLICATIONS

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- I. **Dudek M**, Abo-Ramadan U, Hermann D, Brown M, Canals S, Sommer WH, Hyytiä P. Brain activation induced by voluntary alcohol and saccharine drinking in rats assessed with manganese-enhanced magnetic resonance imaging. *Addiction Biology*. 2014. doi: 10.1111/adb.12179.
- II. **Dudek M**, Canals S, Sommer WH, Hyytiä P. Modulation of nucleus accumbens connectivity by alcohol drinking and naltrexone in alcohol-preferring rats: A manganese-enhanced magnetic resonance imaging study. *European Neuropsychopharmacology*. 2016. doi: 10.1016/j.euroneuro.2016.01.003.
- III. **Dudek M**, Hyytiä P (2016). Alcohol preference and consumption are controlled by the caudal linear nucleus in alcohol-preferring rats. *European Journal of Neuroscience*, accepted manuscript. 2016. doi: 10.1111/ejn.13245.
- IV. den Hollander B*, **Dudek M***, Ojanperä I, Kankuri E, Hyytiä P, Korpi ER (2014). Manganese-Enhanced Magnetic Resonance Imaging Reveals Differential Long-Term Neuroadaptation After Methamphetamine and the Substituted Cathinone 4-Methylmethcathinone (Mephedrone). *International Journal of Neuropsychopharmacology*. 2014; 18(6). doi: 10.1093/ijnp/pyu106.

* Equal contribution

The article **IV** has been used as a part of a previously published dissertation, entitled “Neuropharmacology and Toxicology of Novel Amphetamine-Type Stimulants” by Bjørnar den Hollander.

ABBREVIATIONS

•HO	hydroxyl radicals
2-DG	2-[¹⁴ C]deoxyglucose
4-MMC	mephedrone
5-HT	serotonin
5-HT ₁₋₇	5-hydroxytryptamine receptor type 1-7
AA	alcohol-preferring Alko Alcohol rats
AC	adenylyl cyclase
AIF	apoptosis inducing factor
AIM-MRI	activity-induced manganese-dependent MRI
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPH	amphetamine
Amy	amygdala
ANA	alcohol-nonpreferring Alko Non-Alcohol rats
ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
BBB	blood-brain barrier
BCB	blood-cerebrospinal barrier
BNST	bed nucleus of the stria terminalis
BOLD	blood oxygenation level dependent
cAMP	cyclic adenosine monophosphate
CFN	[¹¹ C]carfentanil
Cg	cingulate cortex
CLi	caudal linear nucleus
CNS	central nervous system
CPP	conditioned place preference
CPu	caudate putamen
CSF	cerebrospinal fluid
CTOP	D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH ₂
D ₁₋₅	dopamine receptor type 1-5
DA	dopamine
d-AMPH	dextroamphetamine
DAT	dopamine transporter
DB	diagonal band
EPSP	excitatory postsynaptic potential
FDA	American Food and Drugs Administration
fMRI	functional magnetic resonance imaging
FOV	field of view
FR	fasciculus retroflexus area
GABA	γ -aminobutyric acid
GABA _{A-B}	γ -aminobutyric acid receptor type A-B
GBCA	gadolinium-based contrast agents
GIRK	G protein-activated inwardly rectifying potassium channel
GP	globus pallidus
HAD	high alcohol drinking (rat line)
HC	hippocampus
HT	hypothalamus
ICSS	intracranial self-stimulation
ICV	intracerebroventricular
iGluR	ionotropic glutamatergic receptor

IHC	immunohistochemistry
INS	insular cortex
IP	intraperitoneal
ISH	<i>in situ</i> hybridization
IV	intravenous
LAD	low alcohol drinking (rat line)
LCGU	local cerebral glucose utilization
LD50	lethal dose 50 per cent
LH	lateral hypothalamus
LS	lateral septum
M1	primary motor cortex
M2	secondary motor cortex
MD	mediodorsal thalamic nucleus
MDMA	3,4-methylenedioxy-methamphetamine
MDMC	methylone
MEMRI	manganese-enhanced magnetic resonance imaging
METH	methamphetamine
mGlu1-8	metabotropic glutamatergic receptor subtype 1-8
mGlu2/3	metabotropic glutamatergic receptor subtypes 2 and 3
mGluR	metabotropic glutamatergic receptor
MnCl ₂	manganese(II) chloride
mPFC	medial prefrontal cortex
MRI	magnetic resonance imaging
MS	medial septum
NA	noradrenaline
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NET	norepinephrine transporter
NMDA	N-methyl-D-aspartate
NMF	nalmeferine
nor-BNI	norbinaltorphimine
NP	alcohol-nonpreferring rats
NTI	naltrindole
NTX	naltrexone
O	orbital cortex
OT	olfactory tubercle
P	alcohol-preferring rats
PAG	periaqueductal grey
PBP	parabrachial pigmented nucleus
Pdyn	preprodynorphin
Penk	preproenkephalin
PET	positron emission topography
PFC	prefrontal cortex
PKA	protein kinase A
PKC	protein kinase C
PKC δ	delta type protein kinase C
PN	paranigral nucleus
PO	preoptic area
POMC	Pro-opiomelanocortin
PTg	pedunclopontine tegmental nucleus
Ra	raphe nucleus

RARE	three-dimensional rapid acquisition-relaxation enhanced
RLi	rostral linear nucleus
RNS	reactive nitrogen species
ROI	region of interest
ROS	reactive oxygen species
S1	primary somatosensory cortex
S2	secondary somatosensory cortex
SuC	superior colliculus
SERT	serotonin transporter
SN	substantia nigra
SNC	substantia nigra pars compacta
sNP	Sardinian alcohol-nonpreferring rats
SNP	single nucleotide polymorphism
SNr	substantia nigra pars reticulata
SNR	signal-to-noise ratio
sP	Sardinian alcohol-preferring rats
SPECT	single-photon emission computed tomography
SC	subcutaneous
SSRI	selective serotonin reuptake inhibitor
STh	subthalamic nucleus
TH	thalamus
TM	transmembrane
VM	ventromedial thalamic nucleus
VP	ventral pallidum
VPL	ventral posterolateral thalamic area
VTA	ventral tegmental area
VTT	ventral tegmental tail
WHO	World Health Organization
ZI	zona incerta
β-FNA	beta-funaltrexamine

ABSTRACT

Alcohol addiction is one of the most prevalent brain disorders in the world. A major hurdle for reducing alcohol-related harms and developing effective treatments is the poor understanding of neural processes responsible for the development of dependence and addiction. Alcohol has been shown to affect various neurotransmitter systems; however, the mesolimbic dopamine (DA) system, which projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), has been thought to play a key role in producing the reinforcing effects of alcohol. The VTA region has also been suggested to be the anatomical site for the interaction of the dopaminergic system with the opioidergic and γ -aminobutyric acid (GABA) systems.

Here, manganese-enhanced magnetic resonance imaging (MEMRI) and behavioral tests were used to study drug-induced alterations in brain activity of the alcohol-preferring AA (Alko Alcohol) and heterogeneous Wistar rats. MEMRI is based on the ability of paramagnetic Mn^{2+} ions to accumulate in excitable neurons, thus enhancing the T_1 -weighted signal in activated brain regions. Mn^{2+} ions can also be transported anterogradely and retrogradely in neurons, released to the synaptic cleft, and taken up by other neurons. These properties allow MEMRI to measure long-term changes in brain activity, as well as map neural pathways involved in acute and long-term drug actions, including drug reward and toxicity.

The AA rats exposed to alcohol compared to water controls displayed a widespread and persistent activation in brain regions that have been previously linked with alcohol reinforcement. Similarly, activity in neural pathways originating in the NAc and projecting caudally to the midbrain was enhanced in alcohol drinking rats. Moreover, this alcohol-induced activation was blocked by systemic naltrexone (NLX) administration. Comparison of naïve AA and Wistar rats revealed a lowered basal activity in the caudal linear nucleus (CLi) of AA rats, which was restored by voluntary alcohol drinking. The intra-CLi γ -aminobutyric acid type A receptor (GABA_A) agonist muscimol produced a dose-dependent increase in alcohol drinking, blocked by co-administration of the GABA_A antagonist bicuculline, suggesting that the CLi GABAergic system is involved in the regulation of alcohol reward. MEMRI was also employed for assessing stimulant-induced toxicity. Methamphetamine and mephedron displayed disparate effects on brain activity, as methamphetamine produced widespread decreases in activity, whereas mephedron increased activity in limited brain areas.

Taken together, the use of MEMRI for mapping alcohol- and stimulant-induced alterations in functional brain activity revealed networks and specific pathways that have potential for guiding further translational efforts to develop medications for drug abuse disorders, as well as for evaluating drug-induced toxicity.

1. INTRODUCTION

Within the European Union, the average alcohol consumption per adult is 11 liters of pure alcohol yearly. Being one of the heaviest-drinking regions in the world, high alcohol consumption is a serious public health concern in the EU. According to recent estimates, 3.4% of the EU population, i.e., 14.6 million people are suffering from the most serious form of alcohol use disorders, alcohol dependence or alcoholism, making it one of the most common psychiatric disorders (Wittchen et al., 2011). Over the years, attempts have been made to reduce alcohol consumption in developed countries by increasing public awareness of the problem, tightening drink-driving policies, reducing availability, increasing prices, and monitoring in smaller communities such as workplaces. Despite all efforts, a significant number of people will be affected by alcoholism, and therefore effective, evidence-based treatments are urgently needed.

Ethanol is known to affect multiple neurotransmitter systems and brain regions. Reductionist research focusing on individual regions and neurotransmitter systems have helped to reveal the basic neural components underlying alcohol actions. However, in order to understand better the neural circuitry mediating alcohol reward during acute and chronic exposure, global mapping methods measuring both structural and functional changes induced by alcohol drinking have been used. Previous approaches used in rodent models include metabolic mapping with autoradiographic deoxyglucose method that measures the rate of cerebral glucose utilization, as well as measurement of expression of immediate early genes such as c-Fos and Egr-1 (Porrino et al., 1998; Vilpoux et al., 2009). In humans and nonhuman primates, the most common approach for clarifying the effects of drugs of abuse has been neuroimaging, employing techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET). All these methods have provided valuable information on the effects of ethanol on the brain.

As neuroimaging is increasingly used also for evaluating the potential and efficacy of pharmacological agents to modulate alcohol-related behaviors (Mitchell et al., 2012), development of neuroimaging tools suitable for preclinical use could increase the translational value of these models. For example, alcohol-related neuronal activation maps could be utilized as translational biomarkers for bridging preclinical animal and clinical research, because they are supposed to closely reflect the underlying neurobiological mechanisms of alcohol effects and their modulation by pharmacological agents. This approach could also be used for evaluating the toxicity resulting from long-term drug exposure. Therefore, in the current series of experiments, we set out with the aim of utilizing the various possibilities offered by MEMRI for studying alcohol-related brain activation and stimulant-induced neurotoxicity.

2. REVIEW OF THE LITERATURE

2.1. WHAT IS DRUG ADDICTION?

Drug addiction, often also called drug dependence, (American Psychiatric Association, 2013) is defined as a chronically relapsing disorder that causes harm to a society and a user by impairing user's health, social life, and productivity. People seek drugs for various reasons, such as curiosity, social pressure, or for its pain and anxiety relieving properties (Marlatt et al., 1988). The development of drug dependence usually starts with the experimentation phase, i.e., the first encounter with the psychoactive effects of a drug of abuse. These drug effects may have reinforcing properties, due to which the drug is taken again and again (White, 1989). Continued drug taking results in the development of tolerance that leads to taking higher doses of a drug in order to obtain the same effects, and finally in dependence revealed by drug-specific withdrawal symptoms, when access to a drug is prevented after prolonged drug use. The neural adaptations underlying alcohol withdrawal may involve decreased brain GABA levels, downregulation of the GABA_A receptor functions, and upregulation of the glutamatergic system (Kohl et al., 1998). During protracted abstinence, the user may experience a negative emotional state, characterized by dysphoria, anxiety, and irritability (Koob and Le Moal, 1997). Desire to avoid this negative emotional state is yet another, and maybe the most powerful, reason for drug taking, referred to as negative reinforcement (Koob, 2004) (Fig. 2.1).

The criteria for substance dependence listed in the Diagnostic and Statistical Manual of Mental Disorders 5th edition, DSM-V (American Psychiatric Association), and in the International Statistical Classification of Disease and Related Health Problems, ICD-10 (World Health Organization) (WHO) include a list of conditions that an individual has to meet to be considered addicted. Even if tolerance and appearance of drug-specific withdrawal symptoms are core features of drug dependence, the term “addiction” is thought to reflect the compulsive features of drug taking, such as taking larger amounts of a drug or over a longer period of time than it was intended, compulsion to seek and take a drug, and continued drug use despite the harms. In this thesis, the terms addiction and dependence will be used interchangeably, and will be also describing animal behavior insofar as there is agreement of the construct validity of the animal models used.

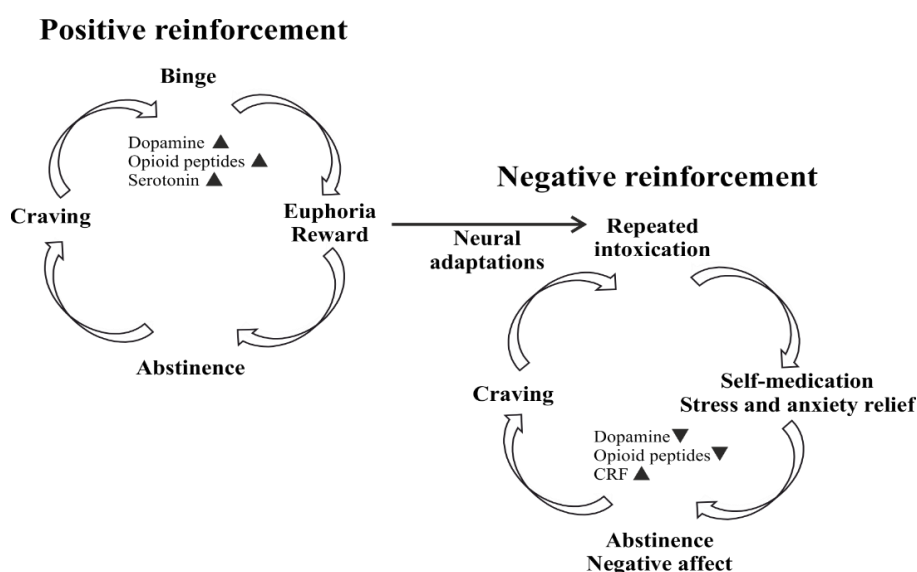


Figure 2.1 Diagram showing a shift from social drinking, driven by positive reinforcement, to dependence and addiction. Binge drinking produces reward and euphoria. After a period of abstinence craving is triggered by alcohol-associated stimuli and the cycle is repeated. Long-term drinking results in specific neuroadaptations that underlie dependence and addiction. Drinking is now motivated by relief from negative affect, e.g. stress and anxiety, which is defined as negative reinforcement.

2.1.1. Alcohol use and addiction

Alcoholic beverages have been an integral part of many cultures for centuries (McGovern, 2009). Prior to the modern era, alcoholic beverages were known only in a fermented form and alcohol was associated with communal activities such as festivals. During early modern industrialization in European countries, new modes of production evolved, leading to promotion and wider distribution of alcohol drinking, as well as to a change in drinking habits (Jernigan, 2000). Discovery of distillation converted alcoholic beverages to a valuable market commodity continuously available during all seasons. This increased availability turned out to be disastrous for the public health (Coffey, 1966). In the 19th century, alcohol was seen as a major impediment to industrial livelihood, and the temperance movements strongly promoted moderate use of alcohol or even sobriety. This was a push for prohibition in numerous countries (Hampel, 1982).

Nowadays, alcohol is the most prevalent addictive substance in the world. According to the most recent WHO data, persons above 15 years drink on average 6.2 liters of pure alcohol per year globally, which translates to 13.5 g of pure alcohol daily (WHO 2014). In Finland in 2013, the total consumption of alcoholic beverages per person aged 15 years or older equaled 12.5 liters of pure alcohol per year. Moreover, 90 % of the population of Finland from the age 15 to 69 reported use of alcohol (National Institute of Health and Welfare, 2015). The prevalence of hazardous alcohol consumption in Finland was estimated at 5.8 % and for comparison, the prevalence of illicit drug use was estimated at 1 % (Halme et al., 2008).

Alcohol, unlike other common drugs of abuse, has been regarded as both a depressant and a stimulant, and has therefore a range of behavioral effects (Fig. 2.2). At blood alcohol levels ranging from 0.1 – 0.5 ‰ (per mille or permil) users experience personality changes, such as increased sociability and talkativeness. There is a general euphoria and increased mood, and also increased confidence and sensitivity to conflict situations. When the blood alcohol level increases to 0.8 – 1.0 ‰, mood swings appear and euphoria interlaces with emotional outbursts. There is also a pronounced release of inhibition. At even higher levels, around 1.5 – 2.0 ‰, alcohol impairs speech, motor skills, and reaction time. Some people experience nausea, vomiting, and blackouts are very likely at this level. At levels of 3.0 ‰, drinkers lose sense of location, may pass out and are difficult to wake up. This state is often referred to as stupor. The lethal dose 50 per cent (LD50) for alcohol is considered to be at the blood alcohol level of 4.0 – 5.0 ‰ in nondependent individuals.

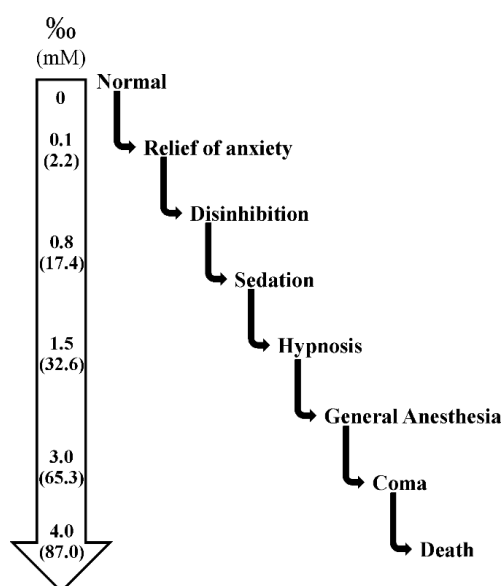


Figure 2.2 Progression of physiological changes corresponding to increased blood alcohol expressed in permils and millimoles.

2.1.2. Designer drugs

The term designer drugs or “novel psychoactive substances” describe newly-misused synthetic psychoactive narcotics that are comparable to classic, previously known illicit substances. Typically, designer drugs are misbranded as “bath salts”, “plant food”, or “research chemicals”. Designer drugs are usually produced in China and South East Asia and cheaply sold world-wide over the Internet (Smith and Garlich, 2013). Slight differences in molecular formulas between scheduled drugs and designer drugs circumvent drug regulations and make them so called “legal highs”.

Designer drugs can be classified on the basis of their effects as stimulants or hallucinogens, or based on their chemical family as amphetamines, aminoindanes, benzofurans, cathinons, phenethylamines, piperazines, pipradrols/piperidines, and tryptamines (Hill and Thomas, 2011). In this dissertation only the amphetamine-type stimulants will be discussed.

2.1.2.1. Natural amphetamine-type stimulants

Amphetamine-type stimulants or amphetamines have been used by humans for centuries. According to some reports, the *Ephedra sinica* plant containing ephedrine, has been found in archeological sites in India and Middle East. Supposedly, this plant was used as an ingredient of a Vedic ritual drink (Mahdihassan and Mehdi, 1989). Clearer evidence from the 1st century AD describes the use of *Ephedra* for medical purposes, mainly as a treatment of asthma and upper respiratory infections (Sulzer et al., 2005). Another example of a natural amphetamine is the *Catha edulis* plant, which is commonly used by chewing its fresh leaves for extended periods. The active compound of this plant responsible for the psychoactive effect is cathinone. It is estimated that the chewing of khat was introduced between 525 AD and the 13th century in the Arabian Peninsula and parts of Africa. Nowadays, in these regions, approximately 80 to 90 % of adult males and 10 to 60 % of adult females chew khat daily (Warfa et al., 2007).

2.1.2.2. Synthetic amphetamine-type stimulants

The first synthetic amphetamine was marketed by Smith Kline and French, under the trade name Benzedrine in 1930s, shortly after its central nervous system (CNS) and respiratory stimulant properties were discovered. The first synthesis of amphetamine, however, was accomplished already in 1887, but its stimulant effects were not noticed (Edeleano, 1887). Effects of Benzedrine were appreciated by various social groups ranging from stay-home-moms to scientists. Therefore, it quickly became one of the most popular and well known drugs of all times. In 1939, in an attempt to reduce Benzedrine use it was scheduled as a prescription-only medicine. By 1970, the pharma industry produced 10 billion tablets, which were being prescribed for treating fever, fatigue, obesity, depression, schizophrenia, and even opiate addiction. It is estimated that up to 90 % of these tablets were diverted to the black market (Rasmussen, 2008a).

Another synthetic amphetamine-like stimulant methamphetamine (METH), was synthesized in 1893 by the Japanese chemist Nagai Nagayoshi (Grobler et al., 2011). Its first marketed form Obetrol was patented by Obetrol Pharmaceuticals in the 1950s, and it was indicated for obesity treatment (Rasmussen, 2008b). METH was also sold in Germany under the trade name Pervitin and was used by German soldiers during WWII to reduce fatigue during long missions. Amphetamines have been used by military forces until present days (Estrada et al., 2012).

Another widely-used drug 3,4-methylenedioxy-methamphetamine, better known as MDMA or ecstasy, was first developed by Merck in 1912, but gained its popularity after it was resynthesized by Alexander Shulgin in 1956. In order to determine the psychoactive properties of the drug he tested it on

himself and described his subjective experience (Shulgin 1995). Due to its properties, the drug was later introduced to psychotherapy in order to increase willingness to communicate. As more and more people became familiar with its positive effects, the US Drug Enforcement Agency scheduled MDMA as a controlled substance in 1985 (Benzenhofer and Passie, 2010).

In the recent years we have seen an alarming growth in the number of new psychoactive substances on the drug black market. Only in the European Union 41 new substances were identified in 2010, 49 in 2011, 73 in 2012, and 81 in 2013 (European Drug Report, 2014). It is estimated that more than 300 new substances were detected by the end of 2014. Amongst the most popular designer drugs are the cathionine substitutes. As mentioned previously, those substances are not covered by existing drug laws due to small differences in molecular formula, making it legal to distribute and sell them over the Internet (Power, 2013).

2.2. NEUROBIOLOGY OF ALCOHOL REWARD

2.2.1. Primary neurobiological targets of alcohol

It was previously believed that the primary targets of alcohol in the CNS are membrane lipids, referred to as the lipid theory. However, the protein theory stating that the primary targets of alcohol are membrane proteins, especially receptors and ion channels (Peoples et al., 1996), has recently gained ground. Today's view is that alcohol has several primary targets, including N-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, GABA_A receptors, glycine receptors, 5-hydroxytryptamine-3 receptors (5-HT₃), and nicotinic acetylcholine receptors (nAChR), as well as L-type Ca²⁺ channels, and G protein-activated inwardly rectifying K⁺ channels (GIRK) (Vengeliene et al., 2008).

NMDA receptors - Electrophysiological studies by Lovinger et al. (1989) showed a concentration-dependent increase in inhibition of hippocampal and cerebellar NMDA receptors by ethanol (Fig. 2.3) (Hoffman et al., 1989; Lovinger et al., 1989). At 50 mM ethanol concentration the current was reduced by 61 % and ethanol's potency in inhibition of NMDA-current was linearly correlated with its intoxicating property. The same phenomenon was later observed in other brain regions, including the VTA, amygdala (Amy), and NAc (Hoffman, 2003). Activation of the NMDA ion channels facilitates influx of cations (such as Ca²⁺) into cells, which was shown to induce NMDA-dependent long-term potentiation (Paoletti and Neyton, 2007). Acute ethanol exposure causes a disruption of cation influx into the cell, thus promoting long-term depression (Blitzer et al., 1990). In 1996, Wright et al. reported that inhibition of NMDA receptors by ethanol does not evoke its inhibitory action on NMDA receptor by changes in fast closed state kinetics, changes in conductance in the open channel, or blockade of the open channel, indicating that ethanol reduces agonist efficacy by allosteric modulation of channel gating (Wright et al., 1996). In cultured cerebellar granule cells, the inhibitory effect of ethanol on the NMDA receptors could be reversed by inhibitors of the protein kinase C (PKC), suggesting a role of the PKC in the ethanol inhibition of the NMDA receptors (Snell et al., 1994). Even though studies have been conducted for decades, a definite binding site of alcohol at the NMDA receptor has still not been identified. Site-directed mutagenesis studies, however, have helped to determine several putative binding sites within the transmembrane (TM) domains 3 and 4 of the GluN1 and GluN2A subunits (Ronald et al., 2001).

AMPA receptors - Ethanol and other straight-chain alcohols have increased potency for inhibition of AMPA receptors comprising the GluA1 or GluA3 subunit (Fig. 2.3) (Akinshola, 2001). Pharmacologically relevant ethanol levels reduce AMPA receptor function by approximately 20 – 25

%. Studies performed on medial septum (MS)/diagonal band (DB) neurons showed that the onset of ethanol inhibition of AMPA receptors is immediate and longer lasting than that of NMDA receptors, where the inhibition diminishes after several minutes (Frye and Fincher, 2000). The subunit composition of AMPA receptors also affects ethanol sensitivity. Thus, receptors containing GluA2 and GluA3 subunits, and receptors containing only GluA3 subunits show less sensitivity to inhibition by alcohol than other combinations (Akinshola et al., 2003).

Kainate receptors - The magnitude of the ethanol-induced inhibition of kainate receptors also depends on the subunit composition. However, in general, quite low ethanol concentrations have been reported to inhibit responses mediated by kainate receptors (Costa et al., 2000). Kainate receptors composed of GluK1 and GluK2 plus GluK4 or GluK5 subunits were inhibited by 5 to 30 % by 25 and 100 mM ethanol concentrations (Costa et al., 2000).

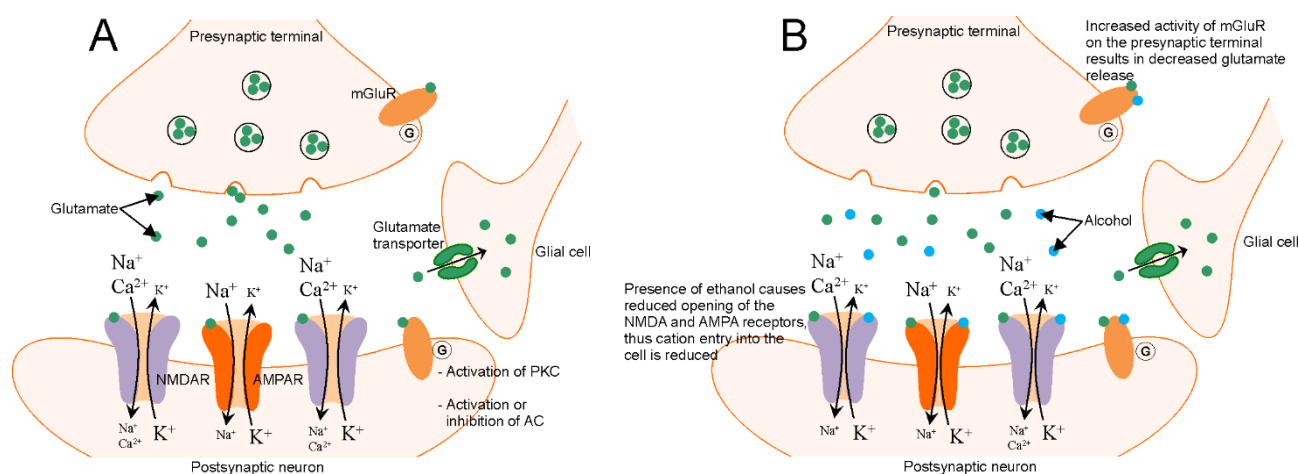


Figure 2.3 (A) Actions of the brain's glutamate system without presence of alcohol. Glutamate (green circles) exerts its effects by acting on NMDA and AMPA ion channels, and G-proteins coupled metabotropic glutamate receptors, thus affecting downstream mechanisms. mGluR, metabotropic glutamate receptor; PKC, protein kinase C; AC, adenylyl cyclase. (B) Actions of the brain's glutamate system after acute alcohol. Acute alcohol (blue circles) reduces the activity of NMDA and AMPA receptors, thus reducing cation entry into the postsynaptic neuron. As a result, fewer nerve signals are generated.

GABA_A receptors - GABA_A receptors have been shown to contribute to various effects of ethanol, such as sedation and anxiolysis, probably by ethanol-induced potentiation of the receptor functions (Lovinger and Homanics, 2007). Each GABA_A receptor is a pentameric ligand-gated ion channel composed mainly of α -, β -, γ -, and δ -subunits (Barnard et al., 1998) and each subunit contains four TM domains forming the chloride channel (Michels and Moss, 2007). Most receptors are composed of $\alpha_1\beta_2\gamma_2$ subunits (60 %) (Sigel et al., 2006). The second and third most common combinations are $\alpha_2\beta_3\gamma_2$ (15 %), and $\alpha_3\beta_3\gamma_2$ (10 %) (Michels and Moss, 2007). Physiological and pharmacological properties of GABA_A receptors, including responsiveness to ethanol, depend on the subunit composition of a particular receptor (Michels and Moss, 2007). For example, GABA_A receptor subunit compositions of $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ are 10 times more sensitive to low concentrations of ethanol (3 – 10 mM) than receptors containing β_2 - instead of β_3 -subunits, and 3 times more sensitive than receptors containing γ_2 - instead of δ -subunits (Wallner et al., 2003). The definite binding site for alcohol on GABA_A receptor has not been characterized. However, a pocket located between TM2 and TM3 of the GABA_A subunit seems to bind alcohol and other anesthetics (Jung and Harris, 2006). Presynaptic GABA release first activates postsynaptic GABA_A receptors, thus inducing phasic inhibition. However, if GABA spillover occurs, also the extrasynaptic receptors are activated inducing tonic GABA inhibition (Fig 2.4). Numerous studies have shown that in some brain regions, including regions of the mesolimbic dopamine system, ethanol can affect

GABAergic transmission through actions at presynaptic, postsynaptic, and extrasynaptic GABA_A receptors (Kullmann et al., 2005).

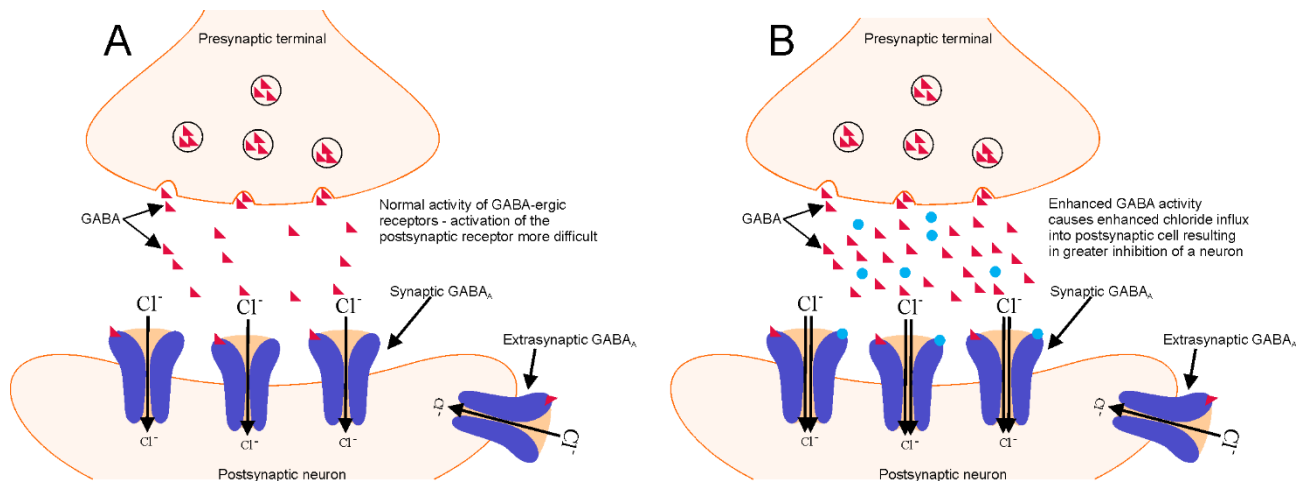


Figure 2.4 (A) Actions of the brain's GABA system without presence of alcohol. GABA (red triangles) acts in part through GABA_A receptors, which are ion channels for Cl⁻ ions. Greater influx of chloride to the postsynaptic neurons makes it more difficult to generate new nerve signals. (B) Actions of brain's GABA system after acute alcohol. Acute alcohol (blue circles) enhances GABA activity resulting in greater influx of chloride ions into postsynaptic neurons and, consequently, their greater inhibition.

5-HT₃ receptors - Various pharmacological manipulations of the serotonin (5-HT) system indicate a modulatory role for 5-HT in alcohol consumption. Ethanol, and other alcohols, have been shown to potentiate the 5-HT₃ receptor function by prolonging the open state and increasing the current mediated by the receptors (Lovinger and Zhou, 1994). In agreement with this view, 5-HT₃ receptor antagonists have been shown to suppress voluntary alcohol consumption in alcohol-preferring rats (Rodd-Henricks et al., 2000a).

nACh receptors - Generally, ethanol has been shown to be a potent modulator of the nACh receptors, especially at low concentrations (100 μM – 10 mM) (Nagata et al., 1996). Nicotinic ACh receptors located on mesolimbic DA neurons play an important role in the regulation of the locomotor activity induced by alcohol consumption (Blomqvist et al., 1992). Moreover, Nisell et al. (1994) reported nicotine-induced DA release suggesting an important role of the VTA nACh receptors in the modulation of the mesolimbic DA (Nisell et al., 1994). Activation of the neuronal nAChRs has also been shown to stimulate GABA release from hippocampal neurons (Alkondon et al., 1997). The nAChR structurally resembles the GABA_A receptor, which also is an important target of ethanol, and similar to GABA_A, responsiveness of nAChRs to ethanol greatly depends on the nAChR subunit composition (Ericson et al., 2003; Le et al., 2000).

Voltage gated L-type calcium channels - Voltage gated L-type Ca²⁺ channels are also primary targets of ethanol. Ethanol reduces the channel opening probability and shortens the open time duration of the channels in a concentration-dependent manner (Wang et al., 1994).

Inwardly rectifying K⁺ channels – Ethanol concentrations relevant to human consumption (18 mM ethanol or 0.8 ‰ blood alcohol level) have been shown to open the inwardly rectifying K⁺ channels, which further inhibits neuronal excitability. A region in the carboxyl terminus of the GIRK has been identified as crucial for the action of ethanol (Kobayashi et al., 1999; Lewohl et al., 1999). It was shown that ethanol affects GIRK channels in cerebellar granule neurons (Lewohl et al., 1999) and midbrain DAergic neurons (Federici et al., 2009).

2.2.2. Brain adaptations to long-term alcohol consumption

During chronic alcohol exposure, continuous activation or deactivation of neuronal membranes induces changes that help to restore equilibrium in the brain and maintain normal receptor functions. Prolonged ethanol induced changes produce tolerance and dependence, which are manifested as decreased behavioral response to ethanol and decreased potency of ethanol to induce intoxication, as well as occurrence of specific symptoms during withdrawal including dysphoria, anxiety, and increased seizure susceptibility (Heilig et al., 2010).

Studies on cultured neurons treated with ethanol in the medium showed that glutamate receptors adapt to long-term inhibitory effects of alcohol by increasing their excitatory activity (Tabakoff and Hoffman, 1996), and upon cessation of alcohol exposure the functions of NMDA receptors are increased in various brain regions, including the cerebellum and cortex (Ahern et al., 1994; Iorio et al., 1992). Moreover, rodent studies showed increased expression of NMDA receptors after chronic alcohol exposure in the hippocampus (HC), amygdala, and cerebral cortex (Floyd et al., 2003; Kalluri et al., 1998). Electrophysiological studies in hippocampal cells demonstrated that more ions were able to pass through NMDA receptors upon opening, and the increased size of dendritic spines suggested presence of higher number of the NMDA receptors (Clapp et al., 2007).

The synaptic AMPA receptors also undergo changes following prolonged ethanol exposure. Neuronal cultures exposed to chronic alcohol were shown to increase AMPA receptor expression in the HC (Bruckner et al., 1997), AMPA receptor function in the basolateral amygdala and VTA (Lack et al., 2007; Stuber et al., 2008), and AMPA receptor-mediated Ca^{2+} flow into the neurons in various brain regions (Clapp et al., 1999).

GABAergic neurotransmission is known to be sensitive to ethanol exposure in numerous brain regions. Prolonged exposure to ethanol has been shown to produce adaptations in the GABAergic system that are reflected in the sedative and anxiolytic properties of ethanol (Kumar et al., 2009). It has been strongly suggested that those adaptations are produced by changes in GABA_A receptor subunit expression in several brain regions (Grobin et al., 1998). For example, the mRNA expression levels of the $\alpha 1$, $\alpha 2$, and $\alpha 3$ subunits were reduced in the cerebral cortex, while $\alpha 4$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$, and $\gamma 2$ subunit mRNA level were increased following voluntary chronic alcohol consumption (Devaud et al., 1997). Chronic ethanol also altered GABA_A subunit expression in other brain regions. For instance, $\alpha 4$ subunit expression was increased in the hypothalamus (HT) for 14 days after chronic alcohol exposure (Devaud et al., 1997), whereas levels of the same subunit were decreased in the Amy and NAc, and in yet other regions, including the HC and VTA, they were not affected (Papadeas et al., 2001). The findings regarding δ subunit expression also vary among brain regions. Chronic intermittent alcohol exposure decreased the expression of the δ subunit in cerebellar and hippocampal cells (Marutha Ravindran et al., 2007). Withdrawal from alcohol, however, caused a decrease in δ subunit expression in the cerebellum and an increase in the HC (Follesa et al., 2006). It is hypothesized that chronic ethanol-induced alterations in the assembly of the GABA_A receptor subunits influence functional and pharmacological properties of the receptors by affecting various mechanisms including protein phosphorylation, regulation of intracellular trafficking, and post-translational modifications (Kumar et al., 2004). Therefore, changes in the subunit composition and not decrease in the number of the GABA_A receptors on the postsynaptic membrane are hypothesized to be responsible for the development of ethanol tolerance and dependence (Grobin et al., 1998).

2.3. BRAIN REWARD SYSTEM

2.3.1. A brief history of the brain reward system research

The brain reward system was first identified by Olds and Milner in 1954. They showed that rats would do everything to either access or avoid electrical stimulation of specific septal nuclei, described as rewarding or aversive (Olds and Milner, 1954). It was later demonstrated that electrical stimulation of the lateral HT would induce arousal and reward (Coons et al., 1965). Intracranial self-stimulation studies (ICSS) became the main tool for identifying brain regions assumed to participate in motivation (Wise, 1974). In 1978, Phillips and Fibiger first demonstrated the role of DA in maintaining brain stimulation in several brain regions, including the medial prefrontal cortex (mPFC), NAc and VTA (Phillips and Fibiger, 1978). Pharmacological manipulation of the relevant brain regions, in particular microinjections of drugs of abuse to the NAc and caudate putamen (CPu), produced reward-related responses. That supported the hypothesis of specific reward circuits in the brain (Carlezon and Wise, 1996). In addition, DA antagonists were found to disrupt learning and behaviors enhanced by stimulants and opiates, suggesting a crucial role for DA in producing responses rewarded by addictive substances (Bozarth and Wise, 1983; Spyraiki et al., 1982). Since then, the dopaminergic system has become one of the main targets in studying the rewarding effects of abused drugs (Pierce and Kumaresan, 2006). Nevertheless, the exact role of DA in drug addiction and reward is still unclear. On one hand, it is hypothesized to mediate euphoria from drugs, but on the other hand it has been linked with the motivation to approach the drug and with anticipation of the possible reward (for review (Berridge, 2007)).

2.3.2. Anatomy of the brain reward system

The brain reward system comprises a widespread neurocircuitry, with several brain regions displaying high sensitivity to electrical stimulation. These brain regions mostly belong to the medial forebrain bundle that connects the VTA DA neurons with the basal forebrain (Fallon and Moore, 1978). However, to fully understand this connectivity the concepts of the A10 dopamine nucleus and VTA region have to be clarified.

The VTA was first acknowledged as a discrete brain region by Tsai (1925) and the word “area” instead of “nucleus” was used to stress the heterogeneous cytoarchitectural features of this region (Tsai, 1925). Later in 1964, Dahlstrom and Fuxe identified locations of monoamine containing cell groups throughout the rat brain. Areas containing dopaminergic neurons, namely A8, A9, and A10, were localized in the ventral midbrain (Dahlstrom and Fuxe, 1964). Only the A10 region, however, was overlapping with the previously described VTA (Dahlstrom and Fuxe, 1964). Nowadays, there are two major concepts defining the VTA. One of them is based on the dopaminergic content rather than cytoarchitectural features and defines the VTA that includes also the midline nuclei, i.e., the interfascicular nucleus, CLi, and rostral linear nucleus (RLi) (Oades and Halliday, 1987). Another concept acknowledges the presence of dopaminergic cell bodies in the midline nuclei, but excludes the midline nuclei from the VTA based on distinct cytoarchitectural features (Swanson, 1982).

According to the first descriptions of the mesolimbic dopamine system, the VTA A10 DA neurons project to the NAc. The nigrostriatal dopamine system, on the other hand, was reported to project from the A9 DA nucleus to the dorsal striatum and olfactory tubercle (OT) (Ungerstedt, 1971). Subsequent studies showed that the VTA A10 DA neurons also project to other limbic regions including the HC, Amy, and prefrontal cortex (PFC) (Fallon and Moore, 1978; Swanson, 1982).

The VTA is a highly heterogeneous brain region which can be divided into four major zones: the paranigral nucleus (PN), parabrachial pigmented nucleus (PBP), fasciculus retroflexus area (FR), and ventral tegmental tail (VTT) (Ikemoto, 2007). The first two nuclei are rich in dopaminergic cell

bodies, whereas the FR and the VTT contain a low density of DA neurons (Swanson, 1982). Previous data suggests that the DA neurons from the VTA project to the ventral striatum with mediolateral topography (Fig. 2.5) (Hasue and Shammah-Lagnado, 2002). The posteromedial VTA and CLi send the majority of the dopaminergic innervation to the medial NAc, whereas the lateral VTA projects primarily to the NAc core and lateral olfactory tubercle (Ikemoto, 2007). In contrast, rostrocaudal topography of the VTA shows little differences in the afferent connections (Geisler and Zahm, 2005).

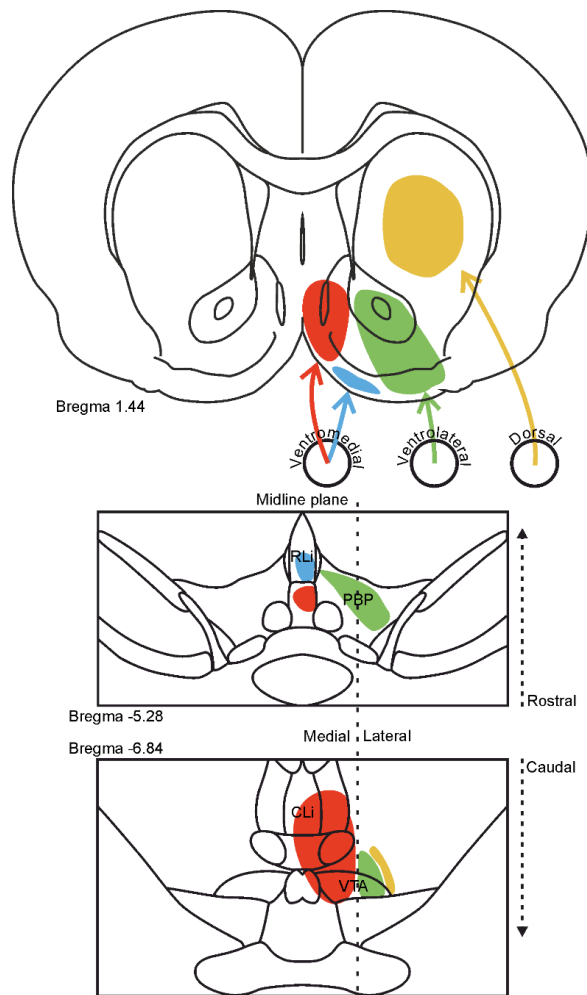


Figure 2.5 Medirolateral topography of dopaminergic projections from the midbrain to striatum. The posteromedial VTA and CLi send the majority of the dopaminergic fibers to the medial NAc, whereas lateral VTA projects primarily to the NAc core and lateral olfactory tubercle. CPu, caudate putamen; CLi, caudal linear nucleus; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; PBP, parabrachial pigmented nucleus; RLi, rostral linear nucleus; VTA, ventral tegmental area.

Other major connections of the VTA include feedback glutamatergic projections from the PFC (Geisler et al., 2007), which target GABAergic neurons projecting to the NAc and DA neurons projecting back to the PFC (Carr and Sesack, 2000). Moreover, recent retrograde tract-tracing studies revealed a large number of previously unidentified brain regions providing substantial glutamatergic inputs into the VTA, including the lateral hypothalamus (LH), preoptic area (PO), bed nucleus of the stria terminalis (BNST), superior colliculus (SuC), periaqueductal grey (PAG), as well as dorsal and median raphe nucleus (Ra) (Geisler et al., 2007). VTA also receives major GABAergic feedback projections from the NAc and ventral pallidum (VP) (Kalivas et al., 1993). Recent optogenetic studies showed that the GABAergic feedback projections from the NAc target VTA non-DA neurons (Fig 2.6) (Xia et al., 2011).

Another dopamine-rich brain region is the substantia nigra pars compacta (SNc). Neurons originating from the SNc project primarily to the CPu creating the nigrostriatal system (Fig. 2.7) (Wise, 2009). Dopaminergic cell bodies located in other relevant regions of the A10 dopaminergic field, such as the RLi and CLi project to the ventromedial striatum, DB, pallidum, and OT (Del-Fava et al., 2007). The majority (about 70 %) of the afferent projections to the SNc are GABAergic. The projections arise mainly from the basal ganglia regions including the CPu and globus pallidus (GP), however part of the innervation comes also from the substantia nigra pars reticulata (SNr) (Tepper and Lee, 2007). Major known glutamatergic projections to the SNc arise from the subthalamic nucleus (STh) and pedunculopontine tegmental nucleus (PTg) (Fig. 2.7) (Bolam et al., 2000).

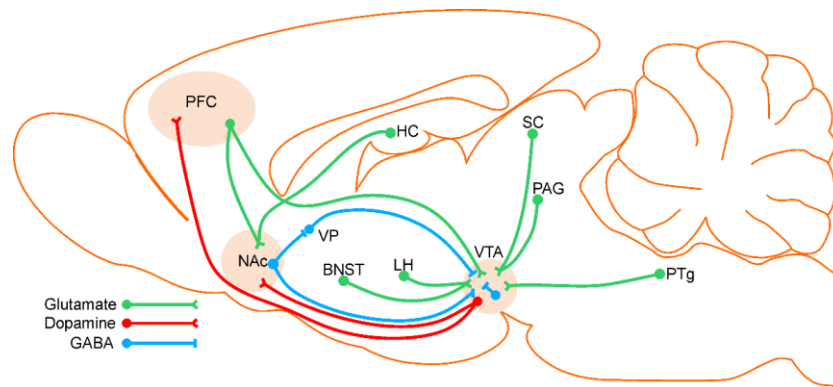


Figure 2.6 Mesocorticolimbic dopaminergic pathway sends dopaminergic efferents from the VTA to the NAc and PFC. The VTA receives feedback afferent GABA projections from the NAc as well as glutamatergic projections from various brain regions. BNST, bed nucleus of the stria terminalis; HC, hippocampus; LH, lateral hypothalamus; NAc, nucleus accumbens; PAG, periaqueductal grey; PFC, prefrontal cortex; PTg, pedunculopontine tegmental nucleus; SC, superior colliculus; VP, ventral pallidum; VTA, ventral tegmental area.

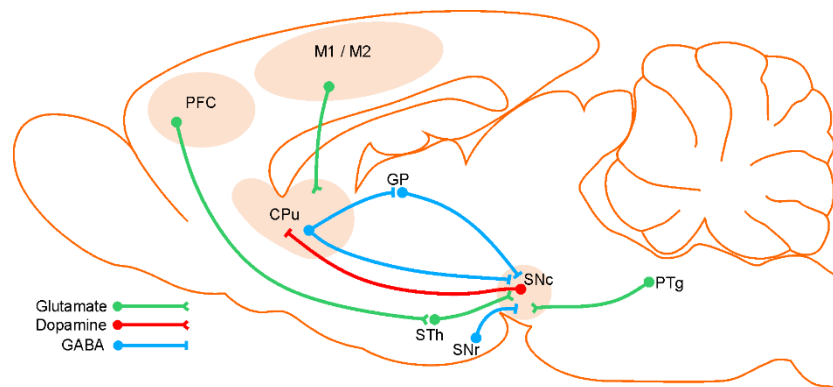


Figure 2.7 The nigrostriatal dopaminergic pathway sends dopaminergic efferents from the SNc to the striatum. The SNc also receives glutamatergic projections from the PFC through the STh. The SNc receives GABA neurons from the CPu and the SNr as well as glutamatergic neurons from the PTg. CPu, caudate putamen; GP, globus pallidus; M1 / M2, primary and secondary motor cortices; PFC, prefrontal cortex; PTg, pedunculopontine tegmental nucleus SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STh, subthalamic nucleus.

2.3.3. Animal models in alcohol research

Animal models remain important tools for studying various phenomena related to alcohol use, such as alcohol-seeking, relapse, tolerance, alcohol-related organ damage, etc.

Animal models attempt to mimic various aspects of human alcoholism. Even though it is not possible to achieve models with complete face validity, there is consensus that partial models with good predictive and construct validity can be built. The main criteria for animal model of alcoholism were proposed in 1979 by Cicero, stating that: an animal should orally self-administer ethanol; the amount of

alcohol consumed should result in pharmacologically significant increase in blood alcohol level; alcohol should be consumed for its pharmacological effect; the consumption of alcohol should be positively reinforcing; chronic ethanol consumption should lead to development of tolerance and dependence; and withdrawal symptoms should occur after access to ethanol is prevented (Cicero, 1979). Later on, an additional criterion stating that animal should also relapse to drinking after abstinence period, was added to the list (McBride and Li, 1998). Commonly used animal models for pharmacological and behavioral studies were produced by directional breeding for high alcohol drinking.

AA and ANA rat lines – The Finnish alcohol-preferring AA (Alko, Alcohol) and alcohol-avoiding ANA (Alko, Non-Alcohol) rat lines, which were used in the present experiments, were developed from a Wistar foundation stock by selective breeding (Sinclair et al., 1989). In a free-choice situation, the AA rats consume from 5 to 7 g/kg/day of absolute ethanol, whereas ANA rats drink less than 1 g/kg/day (Sommer et al., 2006). During forced ethanol drinking, both lines drink identical amounts, but when the free-choice situation is reinstated, the ANA rats immediately drop their intake to 1 g/kg/day or even lower (Sarviharju et al., 2001). In addition to high voluntary alcohol drinking, the AA and ANA rats also show bi-directional changes from heterogenous Wistar rats regarding some behavioral traits such as impulsivity (Moller et al., 1997). Alcohol drinking by AA rats, but not ANA rats, results in increased metabolic tolerance (Forsander and Sinclair, 1992), and tolerance to the motor-impairing and hypnotic effects of ethanol develops faster in the AA rats than ANA rats (Le and Kiianmaa, 1988). During limited access alcohol drinking, AA rats were shown to achieve pharmacologically significant blood and brain alcohol levels (Nurmi et al., 1999). Although AA rats have been initiated to self-administer alcohol using the common initiation techniques (saccharine or sucrose fading) (Files et al., 1997; Hyytia and Kiianmaa, 2001), naïve AA rats were shown to quickly learn to operantly self-administer ethanol without any initiation during continual access to ethanol (Hyytia and Sinclair, 1989).

P and NP rat lines – The alcohol-preferring P and alcohol non-preferring NP rat lines were also developed from a Wistar foundation stock (Lumeng et al., 1977). The P rats consume more than 5 g/kg/day of absolute ethanol, whereas the NP rats do not reach 1 g/kg/day (Li et al., 1987). Importantly, the P rats reach pharmacologically relevant blood alcohol level in the limited and non-limited access paradigms (Bell et al., 2006; Li et al., 1987). Regarding other important criteria for an animal model of alcoholism, P rats were shown to self-administer ethanol under operant conditions, exhibiting high response rates (Penn et al., 1978) and high ethanol intake levels (Murphy et al., 1989). Naïve P rats easily learn to self-administer ethanol and do not need to go through any initiation procedure (Rodd-Henricks et al., 2000b). P rats develop dependence after prolonged free-choice drinking (Waller et al., 1982) and they also display an alcohol deprivation effect (Rodd-Henricks et al., 2001).

sP and sNP rat lines – The Sardinian sP and sNP were also developed from a Wistar foundation stock (Colombo, 1997). Similar to previously described lines, the sP rats readily self-administer over 5 g/kg/day of absolute ethanol under free-choice conditions (Fadda et al., 1989). sP rats attain pharmacologically relevant blood alcohol levels during free-choice drinking (Colombo, 1997). Moreover, sP rats show increased locomotor activity after administration of low alcohol doses, and following limited access alcohol drinking (Agabio et al., 2001; Colombo et al., 1998).

The selected rat lines described above are examples of animal models of alcohol drinking and preference. There are however, a wide variety of animal models used for different aspects of alcohol use disorders, such as animal models of relapse. The best model to study relapse is the reinstatement paradigm (Sanchis-Segura and Spanagel, 2006); however, it poorly mimics the relapse in humans, as humans usually do not undergo the extinction phase preceding reinstatement. Another widely used

animal model of alcohol addiction is the conditioned place preference paradigm (CPP) (Sanchis-Segura and Spanagel, 2006). This model also does not directly mirror human drug seeking. Instead, it utilizes Pavlovian conditioning to assess rewarding effects of alcohol based on comparison of the time spent in the drug-paired environment versus in the nondrug-paired environment following repeated pairings, and therefore provides significant information on the neural mechanisms of drug-environment associations.

2.3.4. Significance of dopamine in the brain reward system

The mesolimbic and nigrostriatal dopamine systems have long been implicated in reward processing in the brain. The VTA DA neurons have been shown to be strongly activated by primary rewards such as food, sex, or drugs of abuse. Upon a rewarding stimulus, the activity of the VTA DA neurons often produce bursts of activity resulting in increased release of DA in the terminal areas, including the NAc and striatum (Di Chiara and Imperato, 1988). Interestingly, DA neurons do not necessarily respond to the reward as such, but instead can also respond to the difference between the received and predicted reward. This phenomena is called the reward prediction error (Schultz et al., 1997). An unanticipated reward induces stronger activation than the anticipated reward (positive prediction error), but if the reward is associated with a predictive cue, repeated presentation of the same cue leads to conditioning, whereby the cue itself induces more DA activation than the reward itself (Ljungberg et al., 1992). However, if the expected rewarding stimulus fails to occur or if the reward is smaller than expected, the VTA DA neurons are inhibited (negative prediction error) (Schultz, 1998; Tobler et al., 2005).

Phasic bursts of DA in response to rewarding stimuli have been hypothesized to induce learning and motivation (Schultz, 2007). Recent optogenetic studies showed that development of the CPP occurs only if the VTA DA neurons are stimulated in a phasic manner (Tsai et al., 2009), whereas if the DA bursting is reduced while the tonic activity remains intact, reward learning is impaired (Zweifel et al., 2009). Conversely to phasic bursts during positive reward prediction error, phasic pauses in activity occur during negative reward prediction error (Bromberg-Martin et al., 2010).

The strict reward-coding hypothesis states that DA is released only in response to rewarding stimuli, whereas aversive stimuli inhibit DA release in the striatum (Roitman et al., 2008). However, DA activity is not restricted to pleasant, or novel and motivational stimuli. Mesolimbic system also phasically responds to non-rewarding stimuli, which can be either aversive or alerting (Ungless et al., 2004). Mesolimbic DA system has been shown to be activated in animals and humans by aversive stimuli such as stress, foot shock, tail pinch, and administration of anxiogenic drugs (Bromberg-Martin et al., 2010). Moreover, recent electrophysiological studies show that DA neurons respond to aversive stimuli in a heterogeneous manner, i.e., they either do not respond or they are stimulated or inhibited (Matsumoto and Hikosaka, 2009). It is possible that induction of DA by aversive stimulation depends on its strength. Strong stimuli have been shown to be more likely than mild stimuli to produce the DA response (Salamone, 1994).

DA responses to alerting events can be triggered by unexpected sensory cues that are interpreted as potentially important based on simple features such as location, size, or movement (Schultz, 1998). In contrast to DA responses to motivational signals, which usually are long lasting, the responses to alerting events are transient and are followed by an appropriate approach or avoidance depending on how the cue will be interpreted by an animal (Schultz, 2010). There are several hypotheses explaining the underlying mechanisms that generate DA responses to alerting signals. One of them states that those responses are simply short lasting reward prediction error signals (Kakade and Dayan, 2002). Recent data, however, suggest distinct mechanisms for generation of DA responses to alerting stimuli (Bromberg-Martin et al., 2010).

2.3.5. Alcohol interactions with various neurotransmitter systems

2.3.5.1. Pharmacological manipulation of the dopaminergic system

Dopamine receptors consist of dopamine D₁-like (D₁ and D₅) and dopamine D₂-like receptors (D₂, D₃, and D₄). High concentration of D₁ receptors overlaps with DA-rich brain regions, including the striatum, SN, and OT. D₅ receptors are mainly found in the HC, thalamus (TH), and cortex (Hall et al., 1994). The D₂ receptors are the most abundant members of the D₂-like family and they are found at high concentrations in DA-rich brain regions, such as the striatum (Hall et al., 1996), and in a bit lower concentrations in the cortex (Sokoloff and Schwartz, 1995). D₃ and D₄ receptors are found predominantly in limbic regions of the brain (Sokoloff and Schwartz, 1995).

Both D₁- and D₂-like receptors subtypes have been implicated in ethanol reinforcement. Studies involving selective agonists of DA receptors showed dose-dependent inhibitory effects on alcohol reinforcement (Cohen et al., 1998; Cohen et al., 1999). Also several antagonists of dopamine D₂₋₄ receptors, such as pimozide or remoxipride, reduced alcohol drinking (Files et al., 1998; Pfeffer and Samson, 1985). Moreover, lower concentrations of D₂ receptors were found in basal ganglia of alcohol preferring AA, sP rat, and P lines than in their counterparts (Korpi et al., 1987). These results suggest that there is a correlation between ethanol preference and the density of the D₂ receptors in brain regions involved in mediation of reward.

Multiple animal studies have shown that systemically (Di Chiara and Imperato, 1985) or locally given ethanol injections (Wozniak et al., 1991) dose-dependently increase extracellular DA concentrations in the NAc. This increase in the striatal DA concentration is probably related with increased firing of the DAergic neurons in the VTA and SN (Brodie et al., 1990; Mereu et al., 1984). Voluntary self-administration of ethanol in the operant conditioning paradigm by alcohol-preferring P rats resulted in the increase in the DA concentration in the NAc (Weiss et al., 1996). Withdrawal from ethanol, on the other hand, decreased the NAc DA levels (Diana et al., 1993).

Ethanol's influence on the dopaminergic system is hypothesized to be at least partly mediated by its interaction with opioidergic and GABAergic systems within the VTA. Acute ethanol was shown to increase levels of β -endorphins in the VTA (Rasmussen et al., 1998), thus stimulating DA release from the dopaminergic terminals within the striatum (Spanagel and Weiss, 1999). The activity of the VTA DA neurons is under constant inhibitory control of GABAergic interneurons, which when active decrease the firing of DAergic neurons. Aforementioned endogenous peptides released after acute alcohol can bind to μ -opioid receptors located on the GABAergic neurons in the VTA and block the inhibitory transmission leading to disinhibition of the DA release (Di Chiara and North, 1992). It has also been shown that ethanol-induced DA release could be mediated through its action on GABA_A receptors in the VTA (Cowen and Lawrence, 1999).

Dopaminergic neurotransmission in the VTA is also partially regulated by cholinergic signaling from the PTg and laterodorsal tegmental nuclei (Garzon et al., 1999). Cholinergic afferents that synapse on the VTA DA neurons have been shown to regulate conditioned DA responses as well as to affect DA phasic signaling (Pan and Hyland, 2005), thus influencing the activity of the mesolimbic DA system. Nonselective nAChR antagonist mecamylamine given systemically was shown to inhibit ethanol drinking in rats as well as ethanol-induced elevation of extracellular accumbal DA release (Ericson et al., 2003). Moreover, the selective $\alpha 4\beta 2^*$ nAChR antagonist DH β E reduced cue-induced elevation of accumbal DA, suggesting that activation of VTA nAChRs is required for cue-induced release of DA in the NAc (Lof et al., 2007).

2.3.5.2. Pharmacological manipulation of the serotonergic system

The 5-HT receptors consist of seven families (5-HT₁–5-HT₇), out of which one family is a ligand-gated Na⁺ and K⁺ cation channel (5-HT₃) and others are G-protein coupled receptors (Hoyer et al., 1994). The 5-HT receptors are widely distributed throughout the brain with the highest concentration in the HC, septum, pallidum, SN, neocortex, and Ra (Baumgarten and Grozdanovic, 1995). The serotonergic system is one of the main targets for treatment of various psychiatric disorders. For example, selective serotonin reuptake inhibitors (SSRIs) are widely used for treatment of depression, obsessive-compulsive disorders, and panic disorders (Nutt et al., 1999). Several 5-HT receptor subtypes, including 5-HT₃, 5-HT_{1A}, and 5-HT_{1B}, have been also shown to play a part in alcohol dependence.

The 5-HT_{1A} receptors are G-protein coupled receptor with seven TM domains (Dohlman et al., 1987). Radioligand binding studies showed a high density of 5-HT_{1A} receptor in several sites of the limbic system including, the Ra, Amy, entorhinal cortex, LS, and HC (Bruning et al., 1989). Studies in selected rat lines demonstrated a significantly higher density of 5-HT_{1A} receptors in the HC and cerebral cortex of the P rats compared to NP rats (Wong et al., 1990). Other studies linked the 5-HT_{1A} receptors also with the alcohol withdrawal symptom (Breese et al., 2005).

Similar to the 5-HT_{1A} receptors, the 5-HT_{1B} receptors are coupled to an inhibitory G protein (Bouhelal et al., 1988). High densities of these receptors are mainly found in the GP, ventral striatum, SN, and dorsal subiculum, and moderate densities in the CPu, HC, PAG, and SC (Bruinvels et al., 1994). The 5-HT_{1B} receptor gene located on chromosome 9 has been linked to alcohol intake phenotypes in mice, and 5-HT_{1B} receptor knock-out mice exhibited increased alcohol intake (Crabbe et al., 1996).

Other elements of the serotonergic system, such as the 5-HT reuptake system, have also been demonstrated to play a role in alcohol addiction, and have been a pharmacological target for the treatment of alcohol dependence. In animal models alcohol exposure increases the serotonin transporter (SERT) mRNA concentration in brain regions where serotonergic cell bodies are located, i.e., the dorsal and median Ra (Oliva and Manzanares, 2007). Preclinical studies have shown that several SSRIs are effective in reducing alcohol consumption, relieving alcohol withdrawal symptoms, and reversing anxiogenic effects of alcohol withdrawal (File et al., 1993). In humans, however, SSRIs mainly improve depressive symptoms in alcoholic patients (Kranzler et al., 1995).

2.3.5.3. Pharmacological manipulation of the GABAergic system

GABA is the major inhibitory neurotransmitter in the brain, regulating many behavioral effects of alcohol drinking and withdrawal (Buck, 1996). GABA receptor subtypes include GABA_A and GABA_B.

As described before the GABA_A receptor is one of the primary targets of ethanol in the CNS, and therefore the GABA_A receptor has been proposed also as a target for pharmacological therapy. Allosteric modulators of the GABA_A receptor that are able to bind to the benzodiazepine binding site are able to block sedative effects of ethanol. The most prominent example of such modulators was the imidazobenzodiazepin Ro 15-4513 that was shown to block the behavioral intoxication produced by ethanol in rats (Suzdak et al., 1986). The drug was not tested on humans because of its proconvulsant and anxiogenic activity. Another candidate with affinity for the benzodiazepine binding site was FG 7142 that suppressed ethanol drinking in alcohol-preferring and -non-preferring rats (June et al., 1998a). The drug was excluded from clinical trials for the same reasons as Ro 15-4513. Several other drugs including flumazenil (aka Ro 15-1788), ZK 93426, and bicuculline were shown to modulate ethanol drinking, but none of them was successful enough to be considered for treating alcohol addiction (Davies, 2003).

Metabotropic GABA_B receptors also have been implicated in addiction disorders. The GABA_B receptor agonist baclofen dose-dependently reduced alcohol intake in alcohol-preferring and Wistar rats

without effects on water consumption (Colombo et al., 2000; Daoust et al., 1987). Baclofen was also shown to diminish the alcohol deprivation effect in alcohol-preferring sP rats (Colombo et al., 2003). The reduction of alcohol-drinking by GABA_B receptor agonists could occur by actions of GABA_B receptors in the mesolimbic dopamine system, as suggested by findings that intra-VTA baclofen infusion blocked heroin self-administration and mesolimbic DA release (Xi and Stein, 1999).

2.3.5.4. Pharmacological manipulation of the glutamatergic system

Glutamate is the major excitatory neurotransmitter in the mammalian CNS, mediating fast excitatory postsynaptic potentials (EPSPs) by activation of ionotropic glutamatergic receptors (iGluRs). Glutamate also binds to metabotropic glutamate receptors (mGluRs), which are coupled to intracellular signal transduction pathways via G proteins (Traynelis et al., 2010). There are three classes of iGluRs: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; subunits GluA1-4), kainate (subunits GluK1-5), and N-methyl-D-aspartate (NMDA; subunits GluN1, GluN2A-D, and GluN3A-B) (Monyer et al., 1994). The iGluRs are found on the dendrites of postsynaptic cells (Traynelis et al., 2010). Thus far, eight members of the mGluRs family have been identified, from metabotropic receptor 1 (mGlu1) to mGlu8. Based on their sequence similarity, relative pharmacology, and signal transduction mechanism they were further grouped into three subgroups (Conn and Pin, 1997). Group I comprises mGlu1 and mGlu5; group II comprises mGlu2 and mGlu3; and group III comprises mGlu4 and mGlu6-8 (Conn et al., 2005). The mGluRs are mainly located in the perisynaptic membranes (Niswender and Conn, 2010).

As described previously, it is well known that alcohol has the ability to inhibit the function of NMDA receptors (Lovinger et al., 1989). Systemic administration of various NMDA receptor antagonists was shown to suppress alcohol drinking in ethanol-dependent rats (Holter et al., 2000). Similar effects were obtained when NMDA antagonists were injected centrally to the NAc (Rassnick et al., 1992) or dorsal striatum (Wang et al., 2007). Some NMDA receptor antagonists were also shown to attenuate cue-induced reinstatement of alcohol seeking (Backstrom and Hyttia, 2004), acquisition of the CPP (Kotlinska et al., 2004), as well as the alcohol deprivation effect (Vengeliene et al., 2005). However, not all tested drugs induced these effects. Similar to NMDA antagonists, AMPA antagonists also attenuate alcohol reinforcement and cue-induced reinstatement of alcohol seeking (Backstrom and Hyttia, 2004). It is important to mention, however, that some of the AMPA antagonists also suppressed reinforcement from sweet solution indicating that those drugs attenuate reinforcing properties of all palatable substances and thus are not specific to ethanol (Backstrom and Hyttia, 2004).

Some genetic studies with knock-out mice also contributed to unraveling the role of iGluRs in mechanisms of action of ethanol. For example, GluN2A knock-out mice do not acquire ethanol CPP (Boyce-Rustay and Holmes, 2006) or develop tolerance to ethanol's hypnotic effects (Sato et al., 2006). GluA1 knock-out mice showed a similar pattern of ethanol consumption as wild-type mice (Cowen et al., 2003), but GluA3 knock-out mice displayed lowered cue-induced reinstatement of alcohol-seeking (Sanchis-Segura et al., 2006).

The Group I family of mGluRs consists of subtype 1 (mGlu1) and subtype 5 (mGlu5). Stimulation of both subtypes of group I mGluRs activates phospholipase C and phosphoinositide hydrolysis (Houamed et al., 1991). The mGlu1 receptors are mostly expressed in the cerebellum, lateral septum (LS), VP, and different thalamic nuclei (Lavreysen et al., 2004), whereas mGlu5 receptors show intense expression in corticolimbic regions (Romano et al., 1995). Only the mGlu5 receptor subtype has been shown to be important in relation to drug actions. Studies examining the role of mGlu1 receptor subtype are very limited. Antagonists of mGlu5 receptor were shown to reduce alcohol seeking and self-administration as well as relapse and rewarding properties of alcohol in mice and rats (Backstrom et al., 2004; Cowen et al., 2005). Moreover, deletion of one of the mGlu5 receptor scaffolding proteins resulted

in reduced alcohol intake and blocked development of alcohol-induced CPP (Szumlinski et al., 2005). In addition, deletion of the mGlu5 receptor blocked development of acute alcohol dependence, increased sedative effects of alcohol, and reduced motor stimulatory effects of alcohol (Blednov and Harris, 2008).

The Group II family of mGluRs consists of subtype 2 (mGlu2) and subtype 3 (mGlu3). Both subtypes are coupled to $G_{i/o}$ proteins and function as autoreceptors regulating presynaptic neurotransmitter release (Pinheiro and Mulle, 2008). The interest in the role of metabotropic receptor subtypes 2 and 3 (mGlu2/3) in the context of the effects of alcohol and drugs of abuse has been growing recently. The most prominent findings in relation to alcohol are that systemic activation of mGlu2/3 receptors reduces alcohol seeking, alcohol self-administration (Backstrom and Hyytia, 2005), and relapse-like behavior, including stress and cue-induced ethanol seeking (Rodd et al., 2006). High expression of mGlu2/3 receptors in limbic brain regions such as the NAc and Amy (Ferraguti and Shigemoto, 2006) could be related to the above behavioral agonist effects, as well as to the modulatory role in expression of the discriminative stimulus effect of alcohol (Besheer et al., 2003).

The Group III family of metabotropic glutamate receptors consists of subtypes 4 (mGlu4) and subtypes 6-8 (mGlu6-8). The group III of mGluR are also coupled to $G_{i/o}$ proteins and activation of them inhibits cyclic adenosine monophosphate (cAMP) formation, thus limiting protein kinase A (PKA) downstream signaling (Mao et al., 2013). The group III has received little attention, but recent data support their importance in drug and alcohol addiction. Most addiction-related research has focused on group III mGluR subtypes 4, 7, and 8, because they are highly expressed in brain regions of the reward circuitry, while subtype 6 is expressed only in the retina, and will therefore not be discussed here (Conn et al., 2005). The Group III mGluRs are located mainly presynaptically. Subtypes 4 and 7 act as presynaptic autoreceptors on the glutamatergic corticostriatal terminals (Conn et al., 2005). They have also been localized on GABAergic terminals connecting the striatum with pallidum and within the SN where they act as heteroreceptors (Kosinski et al., 1999). Subtype 8 mGluRs are expressed in the cortex and striatum (Messenger et al., 2002). Only mGlu7 receptors have been shown to be also located postsynaptically in striatal neurons, but their distribution is not clear (Kosinski et al., 1999). Regarding alcohol abuse, most attention has been focused on the mGlu7 receptor subtype because of its highest concentration in the basal ganglia compared to other subtypes (Dingledine et al., 1999) and also reports linking this subtype to heritable alcohol drinking (Vadasz et al., 2007). The mGlu7 receptor agonist AMN082 decreased ethanol consumption without affecting ethanol metabolism, whereas the blockade of mGlu7 receptors with MMPIP increased alcohol consumption and preference (Salling et al., 2008). Of the other Group III mGluRs, also mGlu4 and mGlu8 receptors have been implicated in alcohol actions. The mGlu4 receptor knock-out mice were shown to have reduced ethanol-induced motor activity compared to wild-type indicating its involvement in mediation of motor stimulant effects of ethanol (Blednov et al., 2004). Systematically administered mGlu8 receptor agonist (S)-3,4-DCPG reduced alcohol self-administration and cue-induced reinstatement of alcohol seeking (Backstrom and Hyytia, 2005).

2.4. OPIOIDERGIC SYSTEM AS A TARGET FOR ALCOHOLISM TREATMENT

Wild animals spend their lives engaging in behaviors that are necessary for their survival, such as searching for food and water, mating and caring for pups, and avoiding predators. They have to constantly adapt to the changing environment in order to survive, therefore those goal-directed behaviors must be flexible (Kelley et al., 2005). These behaviors have generally positive and rewarding emotional outcomes, thereby increasing the probability of the animal behaving in a particular fashion. This phenomenon can be defined operationally as positive reinforcement (Everitt and Robbins, 2005). Some neurotransmitter systems in the brain are hypothesized to be involved in processing positive reinforcing effects (Olds and Milner, 1954). As mentioned above, one of the neurotransmitter systems involved in

this phenomenon is the dopaminergic system, especially the mesolimbic DA system projecting from VTA to forebrain regions including the striatum, Amy, and PFC (Everitt and Robbins, 2005). Nevertheless, many arguments have been presented against DA being a direct and sole mediator of positive reinforcement (Salamone et al., 2007). For example, many lines of evidence support the involvement of the endogenous opioid system in the reinforcing effects of ethanol consumption (Koob et al., 1998).

The existence of opioidergic compounds, such as opium, has been known for centuries, but opioid binding sites in the brain were not established until in 1973 (Pert and Snyder, 1973). There are three main classes of opioid receptors, μ -, δ -, and κ -opioid receptors, and they all have been cloned and sequenced (Akil et al., 1998). Endogenous ligands of opioid receptors, β -endorphin, enkephalins, and dynorphins are produced by proteolytic cleavage of larger precursor proteins known as proopiomelanocortin (POMC), preproenkephalin (Penk), and preprodynorphin (Pdyn) respectively. Enkephalins and dynorphins interact mainly with κ - and δ -receptors, but they also bind to μ -receptors, albeit with lower affinity. β -endorphins bind only to μ - and δ -receptors with almost equal affinity (Akil et al., 1998).

2.4.1. Distribution of opioid receptors in the Central Nervous System

Various ligand autoradiography studies in rodents have helped to determine the distribution of μ -, δ -, and κ -opioid binding sites (Mansour et al., 1995). The highest expression of opioid receptors occurs in the cortex, limbic system, and brain stem. In most of these brain regions expression of all three opioid receptors overlap, but also receptor-specific expression patterns can be detected. Brain regions with high μ -receptor expression include the Amy (excluding the central nucleus), TH, midbrain, and several brain stem nuclei. The κ -receptors are mainly present in the forebrain regions, including the endopiriform cortex, CPu, NAc, PO, and HT. Finally, the δ -receptors are found in the olfactory tract, neocortex, Amy, and striatum. Distribution of the δ -receptors is therefore more restricted, whereas μ - and κ -receptors coexist in most structures (Mansour et al., 1995).

2.4.2. Distribution of neuronal fibers and cell bodies containing and expressing opioid peptides

Mainly immunohistochemistry (IHC) and *in situ* hybridization (ISH) studies have been used to assess the distribution of neuronal fibers and cell bodies that contain opioid peptides. In most cases sites of opioid peptide protein synthesis overlap with localization of opioid receptors. Cell bodies expressing Penk are the most abundant in comparison to other opioid peptide precursor proteins. The highest concentrations of Penk are detected in the TH, where it overlaps with μ -receptors. The highest concentration of Pdyn is found in the NAc, but it is present in many other brain structures, including the HT where it matches the expression of the κ -receptors. POMC is slightly different than other precursor proteins, because its distribution is more restricted. The brain regions containing POMC expressing cell bodies include the arcuate nucleus of the HT, anterior lobe and intermediate lobe of the pituitary, and the nucleus tractus solitarius. These neurons project to the limbic region, midbrain and brain stem, all of which show high immunoreactivity for POMC (Mansour et al., 1995).

2.4.3. Selective ligands of opioid receptors

Opioid peptides and receptors have been shown to play a key role in processing of reinforcement. In general, systemic μ -receptor agonist administration, and to a smaller degree, δ -agonist administration produces positive reinforcement. κ -receptor agonists, on the other hand, induce dysphoria. Systemically

given antagonists of μ - and δ -receptors have been shown to suppress the positive reinforcement produced by natural reinforcers, whereas antagonists of κ -receptors reduced ethanol-induced induction of dysphoria (Van Ree et al., 2000).

2.4.3.1. Effects of selective μ -opioid receptor ligands on ethanol consumption

Several selective antagonists of μ -receptors, including β -funaltrexamine (β -FNA), naloxonazine, and D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), have been shown to reduce alcohol drinking in different experimental paradigms. When injected systemically β -FNA (5.0-20.0 mg/kg) dose-dependently reduced alcohol intake with no effect on water consumption in the high alcohol drinking (HAD) and Wistar rats (Stromberg et al., 1998). Intracerebroventricular (ICV) microinjections of CTOP (0.3-3 μ g) decreased ethanol consumption in alcohol-preferring AA and Wistar rats in the operant paradigm (Hyytia and Kiianmaa, 2001). Systemic administration of CTOP has also been shown to diminish alcohol drinking in C57BL/6 mice (Kim et al., 2000). Naloxonazine (1-15 mg/kg), a long-lasting and irreversible antagonist of μ -opioid receptors, dose-dependently decreased both ethanol and food intake even if injected 20 hours prior to ethanol and food access. Naloxonazine injected prior to three consecutive sessions reduced alcohol drinking only during the first session, suggesting quick development of tolerance to the drug's effects (Honkanen et al., 1996).

2.4.3.2. Effects of selective δ -opioid receptor ligands on ethanol consumption

Although δ -opioid receptor antagonists have generally been shown to exert effects similar to μ -opioid receptor antagonists, the results are less consistent. In the very first report, the δ -antagonist ICI 174864 (0.5-3 mg/kg) dose-dependently reduced ethanol but not water intake in HAD rats in the limited alcohol and water access paradigm (Froehlich et al., 1991), followed by demonstration of reduced alcohol and saccharine drinking by a highly potent and selective δ -antagonist naltrindole (NTI) in rats and mice (Krishnan-Sarin et al., 1995a; Le et al., 1993). Several other studies in mice and rats have reported attenuation of ethanol consumption by NTI administration (Hyytia and Kiianmaa, 2001; Kim et al., 2000). However, these findings have not been consistently replicated by all studies (Stromberg et al., 1998). Yet another δ -antagonist naltriben, which also acts as a κ -agonist, was shown to suppress ethanol drinking in alcohol-preferring rats of the P line (Krishnan-Sarin et al., 1995b), without effects on ethanol discrimination (Mhatre et al., 2000).

2.4.3.3. Effects of selective κ -opioid receptor ligands on ethanol consumption

In contrast to μ - and δ -receptors, activation of κ -opioid receptors produces dysphoria instead of reinforcement. In accordance with this view, the selective κ -opioid receptor agonist U50,488H (2.5, 5.0 or 10 mg/kg) was shown to dose-dependently decrease alcohol intake in rats with free choice to ethanol solution (10 % v/v) and water. The effects of the highest dose (10 mg/kg) of the U50,488H were attenuated by pre-treatment with the κ -antagonist norbinaltorphimine (nor-BNI) (Lindholm et al., 2001). U50,488H was also shown to block ethanol reward and ethanol-induced locomotor activity (Logrip et al., 2009).

The first report on involvement of κ -antagonists in ethanol action showed that systemic administration of a selective κ -receptor antagonist MR-2266-BS (0.1, 1, 5, and 10 mg/kg) unexpectedly reduced ethanol consumption and preference up to three days following forced ethanol drinking (Sandi et al., 1990). The same studies also showed that administration of the MR-2266-BS (1 mg/kg) did not affect the acquisition of CPP when the drug was given before the conditioning (Sandi et al., 1990). In contrast, Mitchell et al. (2005) showed that a single injection of nor-BNI (10 mg/kg) induced a long-

lasting increase in ethanol intake in male Lewis rats that were learning to self-administer ethanol (Mitchell et al., 2005). It has also been shown that the effects of nor-BNI on alcohol drinking may depend on gender, age (adolescents vs. adults), and the state of alcohol dependence of the animals (Morales et al., 2014).

2.4.4. Non-selective ligands of opioid receptors

2.4.4.1. Preclinical data

Naltrexone (NTX) (Fig. 2.8) has been shown to dose-dependently reduce ethanol drinking and preference in various animal models. At doses of 1 – 3 mg/kg naltrexone significantly reduces the palatability and consumption of 10 % ethanol in rats. However, doses as low as 0.1 – 0.6 mg/kg are also effective if NTX is injected shortly prior to drinking and the alcohol access period is short (Koistinen et al., 2001; Stromberg et al., 2001). Much higher doses of NTX are needed if there is a delay between NTX injection and alcohol drinking (June et al., 1998b). NTX was also shown to reduce ethanol reinforcement in other species, including mice and rhesus monkeys (Middaugh et al., 1999; Williams et al., 1998). Intermittent treatment with low and moderate doses of NTX attenuated alcohol deprivation effects in rats with long-term ethanol drinking, but higher NTX doses (10 mg/kg) also suppressed water intake (Holter and Spanagel, 1999). In several studies, NTX also caused a significant decrease in sweetened solution consumption when the sweetened solution was available together with ethanol (Gardell et al., 1996). Effects of NTX have been shown to wear out relatively quickly after administration (Davidson and Amit, 1997), and therefore prolonged delivery systems, such as subcutaneous (SC) NTX pellets and osmotic minipumps have been examined (Holter and Spanagel, 1999; Nation et al., 1990). However, continuous NTX administration either had no effect, suggesting development of tolerance, (Holter and Spanagel, 1999; Nation et al., 1990) or lead to increased ethanol consumption (Phillips et al., 1997). The mechanism of this outcome is hypothesized to be a quick upregulation of opioid receptors (Cowen et al., 1999). Development of tolerance to NTX after repeated daily injections has also been reported (Boyle et al., 1998).

Nalmefene (NMF) (Fig. 2.8) is a newer μ - and δ -opioid receptor antagonist and a potential κ -receptor partial agonist according to findings from cell lines (Bart et al., 2005). Nalmefene structurally resembles naltrexone, but it is superior in several aspects, such as lack of dose-dependent liver toxicity (Mason et al., 1999), higher bioavailability, and longer half-life (Dixon et al., 1987). NMF has been proposed to have advantages over NTX in the treatment of alcoholism (Mason et al., 1999). According to preclinical data, the benefits of NMF have been suggested to arise from its κ -agonist functions, but this view is not very well substantiated. Involvement of the κ -opioid system has been previously reported to play a role in the increased consumption of ethanol in dependent rats. For example, low doses of NMF decreased ethanol self-administration in dependent rats, whereas nondependent rats were not affected (Walker and Koob, 2008; Walker et al., 2011). Intra-accumbal NMF injections were shown to reduce ethanol consumption in dependent and nondependent animals, but the drug was more potent in the dependent animals probably due to upregulation of the NAc dynorphin levels caused by chronic ethanol (Nealey et al., 2011).

2.4.4.2. Clinical findings

NTX was approved for the treatment of alcoholism by American Food and Drug Administration (FDA) in 1994 only after two clinical trials. The first of these trials reported a significant decrease in relapse rate in patients that received 50 mg NTX daily for three months. In addition to decreased relapse rates, alcohol craving was also significantly lowered (Volpicelli et al., 1990). The second three-month study

reported that 50 mg daily NTX significantly reduced alcohol consumption. Moreover, the highest rate of abstinence was obtained when the pharmacotherapy was combined with motivational therapy (O'Malley et al., 1992). Although the majority of the clinical trials conducted later reported positive effects of NTX in treating alcoholism, several, including two biggest ones, showed no differences between NTX and placebo (Kranzler et al., 2000; Krystal et al., 2001). Failures could be ascribed to small sample sizes and short durations of the trials, but the most common reason for treatment failure with NTX was a lack of compliance. Major trials demonstrated that NTX was beneficial only in patients who were compliant with the medication on at least 70 % of days of the treatment (Chick et al., 2000; Monti et al., 2001; Volpicelli et al., 1997). Moreover, other studies suggest that NTX treatment is more effective in patients with a μ -opioid receptor (OPRM1) gene polymorphism (Oslin et al., 2003). Particularly, a single nucleotide polymorphism (SNP) in exon 1 of this gene (118A>G) causes a substitution of asparagine with aspartate at position 40 of the amino acid sequence (Asn40Asp) of the μ -opioid receptor, resulting in increased affinity and potency of β -endorphin at such receptors (Bond et al., 1998). An association between alcohol dependent patients carrying the G allele and the effects of NTX in reducing alcohol-induced stimulation and craving (Ray and Hutchison, 2007), as well as relapse to alcohol drinking (Oslin et al., 2003) has been found.

NMF double-blind, placebo-controlled trials focused on various aspects of alcoholism, such as the number of drinks per drinking day, percentage of abstinent days, as well as on adverse effects produced by different doses of NMF. The first three trials, which all lasted 12 weeks, reported significant decrease in the number of drinks per drinking day in alcohol-dependent patients after 10 to 80 mg NMF compared to placebo. Heavy drinking, defined as 6 or more drinks consumed per day for men and 4 or more drinks per day for women, was significantly decreased only after 40 mg and higher NMF doses (Anton et al., 2004; Mason et al., 1994; Mason et al., 1999). Anton et al. (2004) also reported that motivational therapy did not produce any significant effects on drinking outcomes (Anton et al., 2004). A Finnish, randomized, placebo-controlled multicenter study reported significant findings on different drinking outcomes in heavy-drinking alcoholics. NMF doses of 10 to 40 mg taken by patients were shown to decrease the number of heavy drinking days. During the follow-up randomized withdrawal period, subjects that received placebo returned to heavy drinking. Some adverse effects including, insomnia, nausea, fatigue, and dizziness were reported (Karhuvaara et al., 2007). Recent trials utilizing “as needed” approach reported a significant reduction in heavy drinking days and decrease in daily alcohol consumption after 18 and 20 mg NMF doses versus placebo. The study lasted 52 weeks and the most commonly reported adverse effects were nausea, dizziness, and fatigue. Mild liver toxicity was also reported in the NMF group (Gual et al., 2013; Mann et al., 2013).

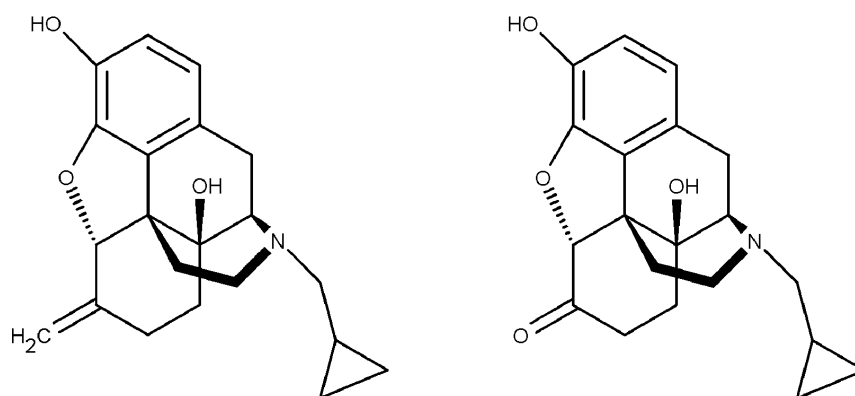


Figure 2.8 Molecular structures of non-selective antagonists of opioid receptors naltrexone (**left**) and nalmefene (**right**). The only molecular difference between these drugs is that naltrexone has a ketone group at the sixth carbon, whereas nalmefene has a methylene group.

2.5. IMAGING IN ADDICTION RESEARCH

Different biochemical, pharmacological, electrophysiological, and behavioral studies have helped to identify how alcohol affects individual neurotransmitter systems at various anatomical sites in the CNS. However, it is highly unlikely that behavioral and physiological effects of alcohol are produced by the actions of single transmitter systems in a specific brain region. In order to understand better neural substrates of ethanol actions it is necessary to investigate ethanol-induced processes in brain circuits and pathways at a global level. To that end, several brain imaging methods have been used, both in experimental animals and humans.

2.5.1. 2-[¹⁴C]deoxyglucose metabolic mapping

The 2-[¹⁴C]deoxyglucose (2-DG) method has been used widely for measuring local cerebral glucose utilization (LCGU) after alcohol exposure (Porrino et al., 1998). For example, experiments using non-selected heterogeneous rats demonstrated increases in LCGU rates in mesolimbic and nigrostriatal regions after low ethanol doses (0.25 g/kg intraperitoneal injections, IP), whereas moderate ethanol doses (1 g/kg IP) significantly decreased LCGU rates in sensory and motor brain areas (Williams-Hemby and Porrino, 1994). Long-term self-administration studies where animals were drinking on average 1.5 g/kg/day reported reductions in LCGU rates in the posterior NAc, HC, and locus coeruleus (Williams-Hemby et al., 1996). Another long-term voluntary alcohol drinking experiment performed on the alcohol-preferring P rats also reported increased functional activity in many brain regions compared to alcohol non-preferring and outbred Wistar rats (Smith et al., 2001a). Moreover, this study showed that after two weeks of alcohol deprivation brain activity in some regions, such as the central and basolateral amygdala, SN, as well as temporal, piriform, and occipital cortices, was restored to the baseline level. However, in other areas it remained completely reduced, including the VTA, PFC, OT, HC, VP, and septum, or was only partially recovered, such as the NAc core and shell, HT, and ventromedial TH. Other reports showed differences in basal LCGU rates between naïve P and NP rats in several limbic regions (Smith et al., 2001b). In contrast, 2-DG studies performed on naïve high-, and low-alcohol drinking rats (LAD) did not find any significant differences in basal LCGU rates in any brain regions examined (Learn et al., 2001).

2.5.2. Manganese-enhanced magnetic resonance imaging

Manganese-enhanced magnetic resonance imaging is a relatively new imaging method that is independent of hemodynamic changes (Silva et al., 2004). Due to its properties, manganese can be used both as a contrast agent and a tracer, allowing description of brain architecture (Eschenko et al., 2010b; Pautler, 2006), mapping of neuronal pathways (Pautler, 2004), and mapping of functionally active CNS regions (Fa et al., 2010). Manganese is essentially a metal that possesses paramagnetic properties, causing reduction of the relaxation time of tissue water protons, and leading to strong enhancement of T₁-weighted MR signal intensity (Silva et al., 2004). After systemic administration, Mn²⁺ reaches the brain via choroid plexus and enters neurons via L-type voltage-gated Ca²⁺ channels and Na⁺/Ca²⁺ exchangers. It is possible because Mn²⁺ acts as a Ca²⁺ analog and both cations have a similar ionic radius (Koretsky and Silva, 2004). Slow efflux of Mn²⁺ ions from the neurons and accumulation in proportion to neuronal activity allow imaging of neuronal activation patterns (Lin and Koretsky, 1997). After local injection, Mn²⁺ is taken up by neurons and transported anterogradely to synaptic terminals, from where it is released to the extracellular space and taken up by other neurons, allowing mapping of neuronal connections (Canals et al., 2008; Pautler, 2004).

Other, less toxic paramagnetic contrast agents are available. The most commonly used are gadolinium-based contrast agents (GBCA) (Sherry et al., 2009). The main downside of gadolinium ions Gd^{3+} compared to manganese is that Gd^{3+} cannot easily cross the BBB (Sherry et al., 2009) and therefore it is mainly used for imaging of brain tumors or lesions that cause a disruption of the BBB and allow influx of the GBCA products into the brain tissue (Hesselink et al., 1986).

2.5.2.1. Activity-dependent assessment of brain function

In studies with small laboratory animals, the main advantage of using MEMRI over PET is its spatial resolution. MEMRI can provide information with approximately 100 μm resolution *in vivo*; whereas PET studies can only reach a resolution in the range of several hundred micrometers (Paul et al., 2009; Tai et al., 2005). Compared with magnetic resonance imaging (MRI) based on the blood oxygenation level dependent responses (BOLD), the advantage of MEMRI is that the hemodynamic changes are short-lived and many important events may be lost when dealing with long-term processes. Another and maybe the most important advantage of MEMRI is that the brain activity revealed by MEMRI is acquired in awake and free-moving animals, which can later be imaged under anesthesia. Anesthesia is required for most *in vivo* animal experiments including MRI studies and the most commonly used anesthetics are urethane, medetomidine, ketamine and isoflurane (Baudelet and Gallez, 2004). Previous reports showed that the depth of anesthesia significantly influences the enhancement of MR signal intensity (Lin and Koretsky, 1997). In MEMRI the great majority of the changes in neural activity are acquired preceding the scanning, and the brief isoflurane anesthesia during the scanning should only minimally affect the results.

On the other hand, a major drawback of MEMRI is both systemic and neurological toxicity produced by high doses of manganese(II) chloride ($MnCl_2$) which excludes the use of this method in human subjects (Eschenko et al., 2010b; Normandin and Hazell, 2002). High doses of $MnCl_2$ are required in order to obtain good MR contrast and signal-to-noise ratio (SNR), and therefore it is crucial to balance the optimal amount of $MnCl_2$ against the desired contrast and the resulting toxic effects. For example, single IP injections of 44 and 66 mg/kg $MnCl_2$ in rats did not produce any behavioral effects, and were therefore considered non-toxic (Berkowitz et al., 2006; Bissig and Berkowitz, 2009), but intravenous (IV) doses as small as 93 mg/kg for rats and 38 mg/kg for mice produced significant adverse effects and mortality rates (Silva et al., 2004). Although changes in MRI signal can be detected with doses as low as 9 mg/kg IV (Lee et al., 2005), substantially higher concentrations are routinely used in order to obtain better contrast (Aoki et al., 2004b; Bock et al., 2008). To avoid toxicity caused by high acute doses of $MnCl_2$ different administration methods have been developed. Bock et al. (2008) reported that six fractionated injections of 30 mg/kg $MnCl_2$ administered systemically at 48 h intervals produced a better signal enhancement than a single dose of 180 mg/kg $MnCl_2$ (Bock et al., 2008). Moreover, the appearance and activity of rats that received fractionated injection was assessed as normal, whereas rats that received one acute injection were described as “ungroomed and lethargic”. Later in 2010, Grunecker et al. conducted a similar experiment in mice and reported that daily systemic injections of 30 mg/kg for 8 days, resulting in a total $MnCl_2$ dose of 240 mg/kg, as optimal for MEMRI contrast (Grunecker et al., 2010). In order to eliminate stress or intrusiveness caused by multiple IP or IV injections interfering with experimental procedures, $MnCl_2$ administration from osmotic minipumps has been developed. They have also been shown to reduce toxic effects of $MnCl_2$. Eschenko et al. (2010a) reported no decrease in locomotor activity of rats after delivery of 80 mg/kg $MnCl_2$ over 7 days (Eschenko et al., 2010a). In contrast, a single $MnCl_2$ injection of as small as 10 mg/kg IV might result in significantly decreased locomotor activity in both non-human primates and rodents (Eschenko et al., 2010a; Gwiazda et al., 2007). Discrepancy of doses and effects they evoke is mainly a result of different routes of $MnCl_2$ administration.

2.5.2.2. Tract tracing

During its short history, MEMRI has been used for tracing neuronal connections *in vivo* in various neuronal circuits. In the pioneering experiment conducted in rats and fish Pautler et al. (1998) indicated that intranasal or intravitreal administration of MnCl_2 leads to MR signal enhancement along the respective neural pathways (Pautler et al., 1998). By delivering MnCl_2 directly into the nasal cavity of mice Chuang et al. (2009) was able to create maps of Mn^{2+} accumulation in the olfactory bulb after exposing animals to different odorant stimuli (Chuang et al., 2009). Enhancement patterns produced by different smells differed from each other, and more importantly, they were consistent with brain areas previously known to be responsible for passing olfactory information. Since it is unknown whether MnCl_2 injected directly to nostrils alters olfactory perception, it was crucial to find a reasonable dose of MnCl_2 that would adequately enhance signal intensity without affecting the smell perception (Chuang et al., 2009). Lehallier et al. (2012) reported that doses in the range 0.3 – 0.8 μmol MnCl_2 did not significantly affect the motor activity or smell perception and the dose of 0.3 μmol MnCl_2 was assessed as the optimal for producing rapid contrast enhancement (Lehallier et al., 2012). Doses of manganese used for intranasal administration are reviewed in Table 2.1.

Table 2.1 Summary of MnCl_2 doses injected into nostrils in MEMRI experiments.

Reference	Year	Concentration (M)	Dose (μmol)	Species
Pautler et al.	1998	0.004 – 3.9	0.002 – 15.6	Mice
Pautler and Koretsky	2002	0.000039	0.000156	Mice
Cross et al.	2004	1	10	Rats
Cross et al.	2006	1	5 – 10	Rats
Drobyshevsky et al.	2006	0.05	2.5	Rabbit
Smith et al.	2007	3.7	14.8	Mice
Cross et al.	2008	1	8 – 10	Rats
Serrano et al.	2008	3.8	7.6	Mice
Chuang et al.	2009	0.01	0.07	Mice
Chuang and Koretsky	2009	0.5	10	Rat
Kivity et al.	2010	0.5	2	Mice
De Groof et al.	2010	0.04	0.4	Bird
Massaad et al.	2010	3.7	7.4	Mice
Peethumongsin et al.	2010	3.7	7.4	Mice
Sharma et al.	2010	3.7	14.8	Mice
Soma et al.	2011	1	10	Rats
Kim et al.	2011	0.16	0.64	Mice
Lehallier et al.	2012	0.1	0.1 – 8	Rats
Gutman et al.	2013	1	5	Mice

Adapted from Lehallier et al. (2012) and modified.

In addition to the olfactory system, the method has also been applied in the visual system to map retinal projections in the rats (Watanabe et al., 2001) and mice (Natt et al., 2002). Yang et al. (2011) used MEMRI to investigate the nociceptive projections of the medial TH in rats (Yang et al., 2011). The method has also been applied in non-human primates, for instance for tracking neuronal connections of the basal ganglia in the macaque monkey (Saleem et al., 2002).

2.5.2.3. Activity-induced manganese-dependent MRI

Due to the property of Mn^{2+} to enter active cells, MEMRI can be used to determine functionally active brain regions after systemic administration. This approach, called activity-induced manganese-dependent MRI (AIM-MRI), was first introduced by Lin and Koretsky (1997). Subsequently, many different experimental protocols have been used to assess brain activity in response to pharmacological

and behavioral manipulation. In the first experiment Lin and Koretsky (1997) used isotonic L-glutamic acid administered to the carotid artery of rats to stimulate the brain (Lin and Koretsky, 1997). This resulted in approximately 200 % increase of the global brain activity. Mn^{2+} ions cross the intact blood brain barrier (BBB) with difficulty, and therefore to detect significant changes in MRI signal upon brain activation the BBB has to be broken with a hyperosmolar agent, for example mannitol, which induces a reversible BBB disruption (Neuwelt et al., 1979; Neuwelt, 1980). However, Mn^{2+} ions are able to slowly diffuse into the brain parenchyma through the blood-cerebrospinal fluid (CSF) barrier (BCB) present in ventricles (Eschenko et al., 2010a). In later AIM-MRI studies it was shown that the signal enhancement was independent of hemodynamic changes (Duong et al., 2000), but was consistent with Fos expression revealed by immunohistochemistry (Morita et al., 2002). AIM-MRI has also been applied to study activation following whisker stimulation in rats (Weng et al., 2007), as well as development and reorganization of the auditory brain in mice (Yu et al., 2005; Yu et al., 2007). Recently, MEMRI has found its application in assessment of brain ischemia and gliosis (Aoki et al., 2003). In brain ischemia a disruption of the Na^+/K^+ pumps causes excessive influx of Na^+ into the cells, which results in opening of Ca^{2+} channels (Pulsinelli, 1992). That leads to enhanced Mn^{2+} influx and accumulation in damaged cells, which can be seen in MEMRI images as a region with significantly higher signal (Aoki et al., 2003; Aoki et al., 2004a). Studies of gliosis with MEMRI are based on the hypothesis that Mn^{2+} accumulation is faster in the reactive astroglia due to the hyperactivity of glial cells, thus producing a stronger MRI signal (Kawai et al., 2010).

2.5.3. Imaging of alcohol-related responses in humans

fMRI – Functional MRI is a non-invasive method of brain mapping with high spatial and temporal resolution. This method is based on blood alcohol level dependent or BOLD signal, which is created by paramagnetic properties of oxyhemoglobin and deoxyhemoglobin in the venous blood (Kwong et al., 1992; Ogawa et al., 1990).

In humans, fMRI studies have focused mostly on alcohol cue reactivity experiments, which compare brain responses to neutral control stimuli with those to drug associated stimuli. The main hypothesis of these studies states that brain activation to alcohol-associated cues is related to their ability to induce craving (Koob and Volkow, 2010). Comparison of cue-induced brain activation between light and heavy drinkers revealed higher amplification of fMRI responses in heavy drinkers in brain areas that belong to motivational circuits, mainly the ventral striatum and insular cortex (INS) (Ihssen et al., 2011). Other brain regions that were shown to positively respond to alcohol-related cues in alcoholic patients were PFC and anterior TH (George et al., 2001). Further studies comparing adolescent drinkers with nondrinkers showed increased activity in limbic and visual brain regions in the drinkers. Moreover, there was a positive correlation between the brain activity within those regions and the number of drinks consumed per month (Tapert et al., 2003). In addition to visual cues, significant increases in brain activity in the mesolimbic areas were demonstrated after alcohol-related olfactory cues in high-risk drinkers (Kareken et al., 2004), and after alcohol taste stimulus (Filbey et al., 2008). However, a meta-analysis of neuroimaging studies of alcohol cue reactivity performed by Schacht et al. (2013a) revealed that activation in these areas may not be significantly different from control subjects. Moreover, they also suggested that regions such as the posterior cingulate and superior temporal gyrus may be enhanced in heavy drinkers compared with controls (Schacht et al., 2013a).

PET and SPECT – PET and single-photon emission computed tomography (SPECT) are quantitative imaging methods that measure radioactivity of radiotracers injected IV (Gardner et al., 1992; Wong et al., 2003). Following of radiolabeling of different molecules, PET and SPECT can be used to measure

various parameters, such as blood flow and volume, glucose metabolism, oxygen utilization, receptor density, affinity, and neurotransmitter release.

PET studies with the DA D₂/D₃ receptor antagonist ligand [¹¹C]raclopride provide evidence for increased endogenous DA release after acute ethanol in humans. These studies are based on the competitive binding of [¹¹C]raclopride to the DA D₂/D₃ receptors, and thus ethanol-induced increase in the endogenous DA results in decreased [¹¹C]raclopride binding (Laruelle, 2000). However, not all [¹¹C]raclopride PET studies have reported consistent results. The first study with oral alcohol administration did not show any effects (Salonen et al., 1997), but decreased [¹¹C]raclopride binding after oral alcohol was later shown in the ventral striatum (Boileau et al., 2003). Another two studies that used IV ethanol infusion during PET also failed to detect any ethanol-induced changes in [¹¹C]raclopride binding (Yoder et al., 2005; Yoder et al., 2007). Next study by the same group however, reported increase in striatal DA concentration after IV ethanol without alcohol-related cues, but alcohol-related cues alone decreased DA concentration in the striatum (Yoder et al., 2009).

Regarding chronic effects of alcohol, various PET and SPECT studies have reported a reduction in the dopamine transporter (DAT) in alcoholics. DAT binding in alcoholics after cessation of long-term heavy drinking was shown to be significantly reduced (Laine et al., 1999b). This finding was later confirmed by Repo et al. (1999) who reported similar findings in type I alcoholics (Repo et al., 1999). Reduction in DAT has been correlated with various symptoms of alcoholism such as depression (Laine et al., 1999a) and novelty seeking (Laine et al., 2001).

PET studies in alcoholics showed a reduction in the number and sensitivity of central D₂ receptors in alcohol-dependent patients, which was probably due to receptor downregulation following chronic alcohol use (Heinz et al., 1996; Volkow et al., 1996). Subsequent PET studies revealed a correlation between alcohol craving and a low rate of dopamine synthesis, as well as reduced availability of the D₂ receptors in the NAc and CPu (Heinz et al., 2004; Heinz et al., 2005b).

Regarding GABA receptors, PET was used to detect decreased levels of the GABA_A/benzodiazepine receptor binding in alcoholics compared to healthy controls in the anterior cingulate cortex (Cg), frontal gyrus, occipital cortex, as well as parietal and temporal cortices (Abi-Dargham et al., 1998). The PET technique also demonstrated increased levels of μ -opioid receptors in the ventral striatum of detoxified alcoholics. Moreover, the availability of the μ -opioid receptors in the regions of the reward system was positively correlated with alcohol craving (Heinz et al., 2005a), confirmed later by Williams et al. (2009). SPECT studies conducted by Heinz et al. (1998) showed a significant decrease in the availability of the serotonin transporter (SERT) in the brainstem of alcohol-dependent patients, which was in agreement with findings obtained with MRI in heavy drinkers (Bloomer et al., 2004). Furthermore, PET studies found that serotonin synthesis was altered in several brain regions in alcohol dependent patients (Heinz et al., 1998; Heinz et al., 2000; Nishikawa et al., 2009).

Various animal studies have indicated the involvement of the μ -opioid receptors in the VTA and NAc in ethanol-induced reward processing and motivation (Kelley, 2004). To test this in humans displacement of the radiolabeled μ -opioid receptor agonist [¹¹C]carfentanil (CFN) was measured with PET after alcohol consumption. Studies of Mitchell et al. (2012) measuring CFN binding following alcohol drinking in heavy drinkers and control subjects reported reduced μ -opioid receptor agonist binding in the NAc and orbitofrontal cortex, suggesting alcohol-induced release of endogenous opioid peptides and involvement of these structures in processing of ethanol reward (Mitchell et al., 2012). Other PET studies reported increased μ -opioid receptor binding in the striatum of detoxified alcoholics with alcohol craving compared to healthy controls (Heinz et al., 2005a). Similar findings were reported when [¹¹C]diprenorphin was used instead of CFN (Frost et al., 1986; Williams et al., 2009).

2.6. TOXICITY OF AMPHETAMINES AND AMPHETAMINE LIKE DESIGNER DRUGS

Due to their chemical structure and similarity with monoamines, amphetamines are able to interact with both the norepinephrine transporter (NET), DAT, and SERT. The main mechanisms of action include reduction of the reuptake and induction of the reverse transport of the endogenous neurotransmitters. Amphetamines also induce release of monoamine neurotransmitters from storage vesicles and can promote the transport of the neurotransmitters to the vesicles (Yamamoto et al., 2010). Amphetamines' properties strongly depend on their chemical structure and the kind of the substitution on their aromatic ring as well as at their α and β carbons of the aliphatic chain. For example, amphetamines without any substitution act as psychomotor stimulants. Amphetamines with a substitution can possess hallucinogenic properties or evoke both hallucinogenic and stimulant actions (Kleven and Seiden, 1992). Amphetamines have been shown to produce various toxic effects including liver, cardio, and nephrotoxicity (Carvalho et al., 2012). Here, however, only the neurotoxic effects of two classical amphetamines, namely amphetamine (AMPH) and METH, will be discussed.

2.6.1. Evidence of amphetamine toxicity

As mentioned before, amphetamines interact with monoamine transporters, thus increasing cytoplasmic concentration of these neurotransmitters and stimulating release of the neurotransmitters from synaptic terminals. In the absence of the monoamine neurotransmitters or when their functions are inhibited the amphetamines do not elicit their effects (Sitte and Freissmuth, 2010).

In animal studies mainly biochemical and histological techniques have been used to evaluate neurotoxic effects of amphetamines. Investigations of humans also include neuroimaging methods. The first studies published in the 70's conducted on animal models described that AMPH produces depletion of monoamine neurotransmitters in the brain of the rats, rabbits, and mice. Later in the 80's, AMPH was also shown to damage DAergic neurons and decrease the activity of tyrosine hydroxylase up to several days after drug administration. Similarly, METH also caused depletion of monoamine levels in the rat brain (for review see (Carvalho et al., 2012)). In addition to the depletion of monoamine brain levels AMPH and METH have been associated with degeneration of neuronal fibers. Continuous dextroamphetamine (d-AMPH) infusion for at least 7 days in rats and mice was shown to produce swelling of DAergic axons in the striatum (Jonsson and Nwanze, 1982). Also METH was found to induce axonal degeneration and swelling of DA neurons in the striatum (Ricaurte et al., 1982).

In non-human primates METH was shown to cause long-lasting depletion of DA in the striatum (Seiden et al., 1988). Rhesus monkeys treated for up to 6 months daily also exhibited depletion in NA levels in all brain areas tested. In some brain regions, including the frontal cortex and midbrain, the noradrenaline (NA) levels remained low, in others however, they returned to normal (Seiden et al., 1976). Also in rhesus monkeys that received SQ METH injections for two weeks, decreases in DA and 5-HT, but not NA levels were observed (Preston et al., 1985). Longitudinal studies in rhesus monkeys reported decreased levels of DA and 5-HT in the striatum and several other brain regions approximately 4 years after the last METH injection. This strongly suggests that METH-induced neurotoxicity might permanently damage the monkey brain (Woolverton et al., 1989).

Human studies on the long-term neurotoxic effects of amphetamines are based on neuroimaging techniques or cognitive tests. Some studies have also examined post-mortem brain tissue of the users. PET studies conducted on healthy humans reported that relatively high euphoric doses of d-AMPH significantly increased the cortical and subcortical cerebral metabolism (Vollenweider et al., 1998). Another study using MRI tested the effects of d-AMPH on motor and cognitive skills. Results obtained 75 min after the d-AMPH administration revealed decreases in brain activity during cognitive tasks in comparison with the baseline (Willson et al., 2004). In analogy to what is seen after AMPH, studies

suggest that METH-induced neurotoxicity may lead to significant changes in brain metabolism and structure, thus leading to impaired cognitive performance (Schwartz et al., 2010). For example, structural MRI studies revealed that abstinent METH users have lower grey matter density in several brain regions including the PFC, which is associated with decreased cognitive performance compared to control subjects (Kim et al., 2006). Such decreases in grey matter density were also found in the striatum, without evidence of decreased cognitive functions (Chang et al., 2005). Functional MRI studies reported decreased activity in METH users in several cortical regions, including the PFC, INS, and Cg during cognitive tasks (Hoffman et al., 2008). Other studies also reported decreased activity in the INS and PFC without consistent effects on actual task performance (Nestor et al., 2011).

2.6.2. Mechanisms of amphetamine toxicity

Mephedron, like other amphetamines, inhibits monoamine reuptake systems (Baumann et al., 2013b). Mephedron is a nonselective inhibitor of membrane monoamine transporters with a potency similar to MDMA (Baumann et al., 2013a). Despite the similarities between MDMA and mephedron they were shown to affect brain monoamine levels differently. For example, mephedron does not elicit striatal astrocyte or microglia activation, as was shown for other amphetamine-like psychostimulants (Angoa-Perez et al., 2012). Moreover, a persistent depletion of cortical and striatal levels of monoamines was reported after short-term repeated doses of MDMA, whereas such effects were not seen after similar mephedron use (Martinez-Clemente et al., 2012). Increase in the brain monoamine concentrations after acute mephedrone is rather rapid. Studies in rats showed that the DA concentration in the NAc after a single injection of 3 mg/kg mephedrone peaked within 20 minutes and returned to normal after 2 hours (Kehr et al., 2011). Studies in rats designed for mimicking human psychostimulant binge treatment revealed that 4 x 10 or 25 mg/kg SC mephedrone injections every 2 hours rapidly decreased striatal DAT and hippocampal 5-HTT function (Hadlock et al., 2011). Other studies (Motbey et al., 2012a) compared effects of 15 and 30 mg/kg IP mephedrone on c-Fos expression and locomotor activity in rats. A particularly strong c-Fos expression by both doses of mephedrone was detected in the cortex, dorsal and ventral striatum and VTA. The results resembled those observed with 2 mg/kg IP METH, which was also used in this study (Motbey et al., 2012a). Regarding locomotor activity, interestingly the lower dose of mephedrone induced a more prominent increase in locomotor activity over 60 minutes after drug administration (Motbey et al., 2012a).

The increase in cytoplasmic concentration of DA and NA is not the only action through which amphetamines evoke their toxic effects on the brain. The major molecular mechanisms involved in amphetamines toxicity are: formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), oxidative stress, mitochondrial dysfunction, excitotoxicity, and one non-molecular but equally important mechanism, hyperthermia (Yamamoto et al., 2010).

2.6.2.1. Hyperthermia

Hyperthermia plays an important role in toxicity of psychostimulants (Yuan et al., 2006). As demonstrated in mice, multiple high doses of METH did not induce depletion of DA neurons in the striatum when the animals were in a cold environment, whereas significant depletion of striatal DA neurons occurred when the animals were in the room temperature (Ali et al., 1996). Similar effects were observed with MDMA (Broening et al., 1995). It is important to note that hyperthermia does not influence DA levels directly. Instead, it is hypothesized that it enhances some reactions initiated by amphetamine treatment (Ali et al., 1996). For instance, it was shown that blockade of METH-induced hyperthermia prevents creation of ROS (Fleckenstein, 1997). Moreover, some neurotransmitter systems have been implicated in mediating the hyperthermic effects of amphetamines. NMDA receptor

antagonists seem to significantly attenuate the depletion of striatal DA and 5-HT (Bowyer, 1995). However, other receptor antagonists, including DA D₁, D₂, and D₃ as well as 5-HT₂ receptor antagonists were shown to either block or limit METH-induced hyperthermia. (Bowyer, 1995).

2.6.2.2. Oxidative stress

Amphetamines have been shown to increase production of ROS and RNS (Fleckenstein et al., 2007), which interact with cellular components. Those interactions damage the cells, a process known as oxidative stress (Catala, 2009). Both acute and repeated METH treatments caused oxidative stress in the cortex, striatum, and HC of rodents (Acikgoz et al., 2001). An increase in the intracellular DA concentration and disruption of the DAT system is thought to be the main mechanism leading to METH-induced oxidative stress. This results in increased production of toxic metabolites of DA oxidation, such as quinones (Fleckenstein et al., 2007). The fact that inhibition of DA synthesis and blockade of DAT reduce METH-mediated oxidative stress supports this hypothesis (Yamamoto and Zhu, 1998). There is also strong evidence linking the delta type protein kinase C (PKC δ) with oxidative stress following METH. In PKC δ knockout mice or in mice that were treated with the PKC δ -inhibitor some oxidative stress indicators were significantly reduced (Shin et al., 2012).

Regarding AMPH, a large, acute dose of d-AMPH increased lipid peroxidation and the generation of hydroxyl radicals (\bullet HO), which were followed by DA neuron depletion in the rat striatum (Wan et al., 2000). Chronic AMPH treatment on the other hand, increased production of thiobarbituric acid reactive substances in the rat cortex and striatum (Frey et al., 2006), and superoxide dismutase in the rat HC (Kuo et al., 2009). The PKC δ was also shown to play a role in AMPH-induced oxidative stress, as suggested by significantly reduced protein oxidation and lipid peroxidation by depletion of the PKC δ gene in both rats and mice (Shin et al., 2012).

2.6.2.3. Mitochondrial dysfunction

Amphetamines reduced adenosine-5'-triphosphate (ATP) stores in the rodent striatum by inhibiting specific enzymatic complexes of the mitochondrial electron transport chain (Burrows et al., 2000). Moreover, single doses of METH or AMPH impaired energy metabolism in rats specifically by decreasing the activity of Krebs cycle enzymes in various brain regions including the Amy, HC, striatum, and PFC (Feier et al., 2012). Amphetamines have also been suggested to induce apoptosis in several regions of the rodent brain. METH induced release of the pro-apoptotic factors, such as apoptosis inducing factor (AIF), Smac/DIABLO and cytochrome c, as well as up-regulated pro-apoptotic proteins and simultaneously reduced anti-apoptotic proteins leading to the recruitment of caspases and initiation of apoptotic cell death (Jayanthi et al., 2004).

3. AIMS OF THE STUDY

Alcohol is the most prevalent addictive substance in the world. Even though considerable effort has been directed towards unraveling its effects on the brain, little is known about the influence of chronic drinking on brain function, mechanisms underlying development of dependence and addiction, and persistence of long-term ethanol-induced alterations of brain function. Similarly, increased use of emerging designer drugs calls for understanding their effects on brain function and cognition.

Therefore, these experiments were designed for assessing the alcohol and stimulant effects on functional brain activity, with an emphasis of novel methodological approaches offered by a translational imaging method, MEMRI.

The aims of the studies were:

- I. To assess global changes in rat brain activation by voluntary alcohol and saccharine consumption using manganese-enhanced magnetic resonance imaging.
- II. To elucidate the role of the nucleus accumbens connectivity in the regulation of alcohol drinking and its suppression by the nonselective opioid antagonist naltrexone.
- III. To determine the neural circuitry underlying the propensity for high alcohol drinking and the modulation thereof by alcohol and GABAergic manipulation.
- IV. To explore how 4-MMC and METH differ in terms of their long-term effects on brain activity and memory function in rats.

4. MATERIALS AND METHODS

4.1. EXPERIMENTAL ANIMALS

The alcohol-preferring AA rat line (University of Helsinki) was developed by directional breeding for high voluntary alcohol consumption in a free-choice situation from a foundation stock of Wistar and Sprague-Dawley strains (Sommer et al., 2006). The most important selection criteria during the early phase of the development included ethanol consumption, ethanol preference, and energy preference. Subsequently the rats were crossed with F1 hybrids from Lewis and Brown Norwegian rats and the ethanol consumption remained as the only selection criterion. Wistar rats were also used in the experiments (III, IV). During all studies (unless otherwise stated) rats were housed individually in either transparent polycarbonated cages (Eurostandard Type IV) (I) or in Individually Ventilated Cages (II, III, IV) with bedding material, wooden stick, and nesting material as enrichment. Rats were housed under controlled temperature ($21 \pm 1^\circ\text{C}$) and relative humidity (55 ± 10 percent) on a 12-hour light/dark cycle (with lights on at 6:00 am). All experimental animal studies were approved by the national Animal Experiments Board of Finland.

4.2. VOLUNTARY ALCOHOL AND SACCHARINE CONSUMPTION (I, II)

Drinking fluids were provided in 250-ml drinking bottles equipped with stainless steel spouts, placed in the food tray of the cage covers. During the first four days, the alcohol drinking rats were given 10 % (v/v) ethyl alcohol as their only drinking fluid to habituate them to the taste of alcohol. Thereafter, rats were allowed a 2-bottle choice between alcohol and water for the next six weeks, during which alcohol consumption reached the plateau. Twice per week, consumption of the fluids and body weights were recorded by weighing, the bottles were filled with fresh solutions, and the left-right position of the bottles was changed to avoid side preference. During this phase, the water-drinking control group had always access to water in two drinking bottles. Similarly, the saccharine-drinking rats were offered a choice between saccharine solution (0.05 % w/v) and water for six weeks. The saccharine concentration was selected so that it would give a saccharine preference ratio close to that of the alcohol solution.

4.3. INTERMITTENT ALCOHOL AND SACCHARINE CONSUMPTION (III)

AA rats were given limited access to 10 % (v/v) ethanol solution or 0.025 % (w/v) saccharine solution with tap water as the alternate choice. Before the first drinking session rats were given only 10 % ethanol or 0.025 % saccharine solution to habituate them to the taste of these fluids. The 2-hour drinking session started at 1100 hours every other day (Mondays, Wednesdays, and Thursdays). The amounts consumed were recorded for each drinking session (± 0.1 ml) and the body weights were recorded weekly. The position of ethanol/saccharine pipettes and water bottles was alternated every drinking session to avoid side preference. The intermittent drinking phase lasted for 7 weeks before the implantation of cannulas (see below) and continued for another 4 weeks during the intracranial microinjections.

4.4. MANGANESE(II) CHLORIDE ADMINISTRATION FROM OSMOTIC MINIPUMPS (I, III, IV)

MnCl_2 was dissolved into Tris-buffered saline (pH 7.4) and administered with osmotic minipumps (Alzet, model 2001) that delivered 200 μl of MnCl_2 (1 $\mu\text{l/h}$) during the seven-day infusion, corresponding to a total MnCl_2 dose of 120 mg/kg/week. The concentration of MnCl_2 in the pumps was

adjusted according to the body weight of the animals. Before the surgery, the pumps were primed overnight in a 37°C saline solution. Animals were anesthetized with a combination of fentanyl/fluanisone and midazolam and the pumps were implanted subcutaneously on the dorsum, slightly caudal to the scapulae. For post-surgical analgesia, animals received a subcutaneous injection of carprofen immediately after implantation. For MRI imaging in study **I**, the alcohol-drinking rats were subdivided into two groups matched for their baseline alcohol intake. The first group continued to drink alcohol during MnCl₂ administration. The second group received the minipumps 1-3 days after discontinuation of alcohol drinking, and had access to only water during MnCl₂ delivery. Therefore, the latter alcohol group shared the history of alcohol exposure with the first group, but did not experience the acute alcohol effects during MnCl₂ administration. All rats were imaged immediately following the seven-day MnCl₂ infusion (**I**). In the third study (**III**) the osmotic minipumps were implanted to the naïve animals and in the last study (**IV**) the osmotic minipumps were implanted a week after the termination of repeated drug injections, and the imaging was therefore performed two weeks after the injections.

4.5. INTRA-ACCUMBAL MANGANESE(II) CHLORIDE ADMINISTRATION (II)

Rats were anesthetized with 5% isoflurane and placed in a stereotaxic instrument with the incisor bar positioned at 3.3 mm below the interaural line. Anesthesia was then maintained with 2% isoflurane during the intracerebral injection and subsequent MRI scanning. Injections of MnCl₂ were aimed at the NAc using the following stereotaxic coordinates chosen with reference to the atlas of Paxinos and Watson (2007): + 1.9 from the bregma, - 1.5 from the midline, - 7.2 from the skull surface (Paxinos and Watson, 2007). The selected MnCl₂ concentration of 100 mM and the injection volume of 50 nl were identical or lower than the previously determined thresholds under which no neuronal cell death or astrogliosis could be seen (Canals et al., 2008). The injections were performed with a 0.5 µl Hamilton syringe with a 26G needle over a period of 40 min, resulting in an infusion rate of 1.25 nl/min. After completing the injection, the needle was left in the brain for 10 min, whereupon it was slowly retracted over a period of 2 min. The drill hole in the skull was closed with bone wax and the scalp was sutured. The acquisition of the baseline MEMRI scans was initiated immediately following the intracerebral MnCl₂ injections.

4.6. SYSTEMIC NALTREXONE ADMINISTRATION IN DIFFERENT EXPERIMENTAL GROUPS (II)

Four experimental groups were used: (1) the group drinking water continually, (2) the group with a history of continual two-bottle choice alcohol drinking withdrawn from alcohol for seven days, (3) the group drinking alcohol continually in a two-bottle choice situation, (4) the alcohol-drinking group injected acutely with a 3 mg/kg dose of NLX. Once intra-accumbal MnCl₂ injections were terminated, all rats were imaged immediately (see above), followed by a second imaging session the next day. For evaluating the effects of NLX on the activity of NAc output projections, alcohol-drinking rats were injected either with saline or NLX 30 min before the onset of the dark period, during which rats had free choice to water and alcohol. The injections were given 3 – 5 hours after the baseline scan, with the interval length counterbalanced between the experimental groups. The second MRI scan was acquired 24 ± 1 hour after the baseline scan.

4.7. MRI DATA ACQUISITION (I – IV)

MRI was performed on a 4.7 T scanner (Bruker, PharmaScan 47/16 US, Ettlingen, Germany) using a 38 mm linear volume coil for transmit and receive. Rats were anesthetized with 5 % isoflurane in oxygen (1 L/min) and secured on a custom-made holding apparatus with a stabilizing tooth bar and a nose cone. During scanning, the isoflurane concentration was maintained at 2 – 3 %, and the body temperature was kept constant with a heating pad. T₁-weighted images were acquired using a three-dimensional rapid acquisition-relaxation enhanced (RARE) pulse sequence (repetition time TR = 300 ms, echo time TE = 12.5 ms, number of averages = 7, number of slices: coronal = 127, sagittal = 57, axial = 57, flip angle = 180°, field of view (FOV) = 26 x 17 x 17 mm, and matrix size = 128/54/54, resulting in 0.2 x 0.31 x 0.31 mm voxel resolution.

4.8. MRI DATA ANALYSIS (I – IV)

The MRI images were converted to Analyze format and scaled up by a factor of 10. The MRI images were spatially preprocessed with custom-developed MatLab functions. In brief, brain-extracted T₁-weighted images were spatially normalized using a representative anatomical image and further co-registered to a stereotaxic rat brain MRI template (Schwarz et al., 2006) by a 12-parameter affine transformation using the FSL/ FLIRT tool (Jenkinson et al., 2002). Co-registration of the template to the digitized Paxinos and Watson (1998) atlas enabled atlas-base generation of region-of-interest (ROI) masks for detailed anatomical analysis (Paxinos and Watson, 1998). The resulting images were smoothed with a 4 x 4 x 4 mm Gaussian kernel at full width at half-maximum to improve signal-to-noise ratio with the exception of study **II**, in which no smoothing was used. For identifying the brain regions in which activation in the experimental groups differed from the control group, preplanned voxel-wise independent or paired t-tests were performed in SPM8 (www.fil.ion.ucl.ac.uk/spm/). Systemic MnCl₂ infusion can lead to inter-individual differences in Mn²⁺ accumulation which is reflected by inter-individual differences in global signal intensity. Therefore, in experiments in which global brain activation was assessed, the mean global intensity was treated as a nuisance factor that was removed on voxel-by-voxel basis using the ANCOVA normalization option in SPM (Friston et al., 1990).

In order to further characterize brain activation differences between experimental conditions, anatomical ROI analyses were performed on brain regions selected on the basis of hypothesized involvement in circuits relevant in a particular study and the observed brain activation patterns. These regions included both individual nuclei and larger composite structures. Three-dimensional ROI masks were created with WFU_PickAtlas toolbox (Maldjian et al., 2003), and the selected ROI masks were applied to previously generated SPM contrast files. Thus, ROI analysis was performed using the SPM design from the global analysis. Normalized mean signal intensity values from individual ROIs were extracted using the REX tool (Duff et al., 2007) and analyzed with appropriate statistical tests.

4.9. OTHER METHODS USED ONLY IN THE STUDY NR III

4.9.1. Intra-CLi cannula implantation

Rats were anesthetized with isoflurane in oxygen (0.8 – 1 L/min) and placed in a stereotaxic instrument with the incisor bar positioned at 3.3 mm below the interaural line. Unilateral 23-gauge stainless steel guide cannulas (10 mm in length) were aimed at the CLi with reference to the atlas of Paxinos and Watson (2007): anteroposterior, – 6.6 mm from bregma; mediolateral, – 2.2 mm from the sagittal suture; ventrodorsal, – 8.2 mm from the skull surface (Paxinos and Watson, 2007). In order to avoid rupturing

of the superior sagittal sinus and the brain aqueduct the cannulas were inserted to the brain at a 16 degree angle relative to the skull surface. The cannulas were secured to the skull and anchor screws by a layer of dental cement. Protective dummies were placed in cannulas to prevent infection and blockage. For postsurgical analgesia animals received injection of carprofen and were placed in home cages for recovery. For habituating the rats to injection procedures, they were handled daily, with unscrewing and removing the dummies.

4.9.2. Intra-CLi microinjections

The drugs used for injections were a selective GABA_A receptor agonist muscimol and a selective GABA_A receptor antagonist bicuculline (Abcam Biochemicals, Cambridge, UK). The drugs were dissolved into sterile saline to desired concentrations of 3, 10 and 30 ng/0.5 µl for muscimol and 3, 10 and 20 ng/0.5 µl for bicuculline, and were infused through 30-gauge injector cannulas connected to 10 µl Hamilton Microliter syringes via polyethylene tubing. The injectors extended 1 mm below the guide cannulas. All injections (0.5 µl) were delivered 10 min before ethanol or saccharine self-administration sessions over a period of 60 sec with a Stoelting Quintessential Stereotaxic Injector (Model No.5311, Stoelting Co. Wood Dale, IL). One-minute diffusion time was allowed before the injectors were retracted from the cannulas. In order to test effects of intra-CLi muscimol on alcohol drinking, each AA rat from this group (N = 7) received a vehicle injection, as well as 3, 10, and 30 ng muscimol injections. In order to evaluate whether muscimol's effects on alcohol drinking could be blocked by bicuculline, a separate group of animals (N = 8) received injections of vehicle, as well as those of 10 ng bicuculline + 10 ng muscimol, and 20 ng bicuculline + 10 ng muscimol. Intervals between consecutive injections for each individual rat were at least 48 hours.

4.9.3. Locomotor activity

Four weeks before the locomotor activity measurement animals were implanted with unilateral 23-gauge stainless steel guide cannulas and handled as described above. For two days prior the first measurement, the animals were habituated to the experimental room and cages used for the testing each day. On the day of the locomotor activity measurement the animals were transported to the experimental room 30 minutes prior the measurement. Subsequently, the animals (N = 9) received intra-CLi injections of saline or muscimol (10 ng/500 µl). Each animal received both the vehicle and drug injection and intervals between the injections for each individual animal were at least 24 hours. The animals were placed in transparent polycarbonate cages (26 x 43 x 18 cm) immediately after the injection, and the locomotor activity measurement started 10 min later. The locomotor activity of the animals was measured continuously for 1 hour using the Ethovision software (Noldus Information Technology) connected to a closed-circuit television camera with an overview of all 5 cages in the test arena. After the measurement animals were returned to their home cages.

4.9.4. Brain collection and histology

After completion of behavioral testing, rats pretreated with intra-CLi injections were anesthetized with 5 % isoflurane and killed by decapitation. The brains were collected, frozen immediately and stored at – 96 °C. To verify the cannula placements, coronal sections were cut at 40 µm intervals through the cannula tract. The slides were stained with 0.1 % thionin solution and evaluated by a light microscope and the rat brain atlas of Paxinos and Watson (1998).

4.10. OTHER METHODS USED ONLY IN THE STUDY NR IV

4.10.1. Drug treatment

In order to investigate the potential neurotoxic effects of mephedrone (4-MMC) and METH, rats were treated twice daily for four consecutive days with 4-MMC (30 mg/kg) or METH (5 mg/kg) as a positive control. Two weeks after the final drug treatments MnCl₂ administration and imaging were conducted as described below.

4.10.2. Behavioral experiments

Locomotor activity and sensitization. Animals were habituated to the cages used in the experiment for 2 hours each day. The test took place in the polycarbonate cages (26 x 43 x 18 cm) similar to their home cages. Animals were placed in the cages 30 minutes prior to the injections in order to habituate them to the novel environment. The locomotor activity was then measured on the first and last day of the drug treatment for up to 6 hours in the novel cages after the first of two daily drug administrations. Data from both days were then analyzed, and sensitization was assessed by comparing the locomotor activity on the first and last day of the treatment (Motbey et al., 2012b).

Novel object recognition. Memory function was assessed using the novel object recognition test (Motbey et al., 2012b) in which the preference for exploring a novel object is used as a measure of recognition memory. For this test animals were habituated to the testing arena, a rectangular opaque blue box (50 x 80 x 30 cm) for two days prior to the day of the test. The test consisted of one 3-minute sample trial where a rat was exposed to two identical objects, followed by a 3-minute test trial that took place 15 minutes later, in which the rat was exposed to one novel object and one object that was present previously. The objects were a coffee mug, and two rectangular plastic bottles taped together. The objects were firmly attached to the bottom of the box in order to prevent animals from moving them. Active exploration of the object, such as touching and sniffing, was counted as investigation, whereas being in the vicinity or perching, was not counted as investigation.

5. RESULTS

5.1. GLOBAL CHANGES IN RAT BRAIN ACTIVITY AFTER VOLUNTARY ALCOHOL AND SACCHARINE CONSUMPTION

5.1.1. Functional mapping of brain activity

In earlier studies global analysis of alcohol effects on the brain has been attempted with metabolic mapping that measures the rate of local cerebral glucose utilization (Porrino et al., 1998), or by detection of inducible transcription factors encoded by immediate early genes, including *c-Fos* and *Egr-1* (Vilpoux et al., 2009). However, these methods may not be able to capture long-term effects of chronic alcohol intake. MEMRI combined with delivery of Mn^{2+} has been previously used for mapping brain activity patterns associated with various behavioral tasks in rats, such as voluntary running, intake of snack food, and responding to acute stress (Bangasser et al., 2013; Eschenko et al., 2010a; Hoch et al., 2013). Therefore, we hypothesized that alterations in neuronal circuits recruited during voluntary alcohol drinking could also be detected by MEMRI.

Statistical parametric maps generated by statistical comparison of the alcohol- and saccharine-drinking rats, as well as the abstinent group with the water-drinking control group showed a significant increase in T1-signal intensity in the experimental groups ($p < 0.01$, corrected). Enhanced T1-intensity reflects accumulation of Mn^{2+} ions into active brain regions and is therefore suggestive of increased brain activity.

5.1.1.1. Long-term alcohol drinking produces a region-specific increase in T₁-weighted signal intensity

During chronic alcohol drinking, activation was observed in many cortical areas, including the M1, primary somatosensory cortex (S1), and parietal cortex. In the PFC, the prelimbic cortex, INS, and Cg exhibited activation clusters. In the subcortical telencephalon, alcohol drinking activated the NAc, CPu, and BNST. The striatal activation cluster extended to the ventral BNST, dorsal regions of the PO, and substantia innominata. Dorsal to this cluster, activation particularly during alcohol exposure was observed in the ventromedial (VM) and mediodorsal (MD) thalamic nuclei, as well as more laterally in the ventral posterolateral thalamic (VPL) area. In the midbrain, alcohol drinking activated parts of the SN and the STh, and the cluster extended to the PAG and reticular TH. This activation cluster also included the PAG, mesencephalic reticular formation (deep mesencephalic nucleus), and SuC (Fig. 5.1).

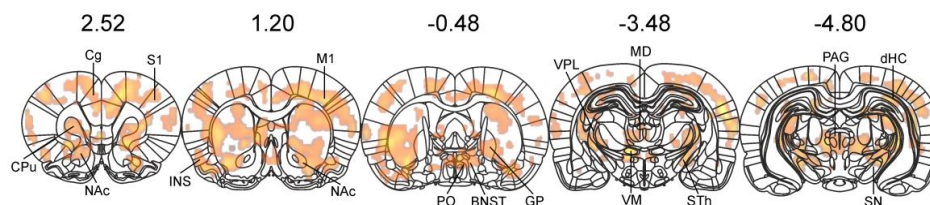


Figure 5.1 Differences in T₁-weighted MRI signal intensity between water and alcohol drinking rats revealed by a voxel-wise independent t-test performed in SMP8. Activation heat maps were manually superimposed on corresponding sections from rat brain atlas (Paxinos and Watson, 2007). Numbers over the sections represent distance from bregma in stereotaxic coordinates. Abbreviations used: BNST, bed nucleus of the stria terminalis; Cg, cingulate cortex; CPu, caudate putamen; dHC, dorsal hippocampus; GP, globus pallidus; INS, insular cortex; M1, primary motor cortex; MD, mediodorsal thalamic nucleus; NAc, nucleus accumbens; PAG, periaqueductal grey; PO, preoptic area; S1, primary somatosensory cortex; SN, substantia nigra; STh, subthalamic nucleus; VM, ventromedial thalamic nucleus; VPL, ventral posterolateral thalamic area.

5.1.1.2. Alcohol-induced brain activation persists for at least a week following withdrawal in several brain regions

During early abstinence, several forebrain areas displayed continued activation, including the Cg and prelimbic cortex, CPu and NAc, but these clusters were smaller than those during alcohol drinking. Similar with alcohol, high activation was also seen in the ventral BNST and preoptic regions.

5.1.1.3. Saccharine drinking induces a pattern of activation similar to alcohol drinking

The brain activation maps induced by saccharine drinking exhibited noticeable similarity with those found during chronic alcohol ingestion. Thus, conspicuous activation clusters were observed in the NAc, CPu, BNST, dorsal HC, and in the cortical regions, including Cg, M1, and S1.

5.1.2. Region of interest analysis

To further analyze the intensity differences between the experimental conditions, intensity values from 30 anatomically defined brain regions were extracted. Instead of voxel-wise analysis, the mean intensity values across the ROIs were extracted and compared. A two-way repeated measures analysis of variance (ANOVA) with treatment as the between-subject factor and brain region as the within-subject factor revealed a significant effect of both exposure ($F_{3,812} = 5.72$, $p < 0.01$), and region ($F_{29,812} = 246.4$, $p < 0.0001$), as well as a significant exposure x region interaction ($F_{87,812} = 26.07$, $p < 0.0001$).

The simple effect analysis showed that alcohol and saccharine induced significantly greater activity compared to control water ($F_{1,406} = 8.64$, $p < 0.01$ and $F_{1,406} = 12.14$, $p < 0.01$, respectively). The abstinence group did not differ significantly from the water condition ($F_{1,406} = 3.97$, $p = 0.066$). There was however, a significant exposure x region interaction ($F_{29,406} = 54.62$, $p < 0.0001$). No significant differences were found between alcohol and abstinence ($F_{1,406} = 0.48$, $p = 0.50$) or between alcohol and saccharine drinking ($F_{1,406} = 1.44$, $p = 0.25$); however, exposure x region interaction was significant in both cases (alcohol vs. abstinence $F_{29,406} = 46.04$, $p < 0.0001$; alcohol vs. saccharine $F_{29,406} = 3.16$, $p < 0.0001$).

Bonferroni post hoc t-tests were performed to directly compare conditions at each ROI included in the overall analysis. Alcohol-induced signal intensity was significantly higher than in the water group in several regions including the IL, NAc shell, septum, S1, GP, LH, Ce, dorsal HC, dorsal and ventral TH, SN, VTA, PAG, and Ra. Differences between the abstinence group and alcohol group were mainly caused by deactivation of few brain regions during early abstinence including INS, M1, septum, LH, dorsal HC, SN, PAG, and Ra. In the early abstinence group however, the prelimbic cortex, ventral HC, and STh showed significantly higher activation than during alcohol drinking. In general, saccharine drinking-induced signal intensity did not differ significantly from the alcohol condition, although slightly higher values were found in the prefrontal regions. Results of the statistical analyses are summarized in Table 5.1.

Table 5.1 Summary of the statistical tests performed on the ROIs.

	Two-way RM ANOVA					
	Exposure		Region		Interaction	
	F	p	F	p	F	P
ROI analyses between all experimental conditions	$F_{3,812} = 5.72$	< 0.01	$F_{29,812} = 246.4$	< 0.0001	$F_{87,812} = 26.07$	< 0.0001
	Simple effect analyses			Bonferroni post-hoc		
	F		p	ROIs with significantly higher activity		
Alcohol vs water	$F_{1,406} = 8.64$		< 0.01	IL, INS, NAcC, MS, LS LH, S1, GP, Ce, HC, TH, SN, VTA, PAG, Ra		
Saccharine vs water	$F_{1,406} = 12.14$		< 0.01	CPu, NAcC, M1, S1, PO, BNST		
Abstinence vs water	$F_{1,406} = 3.97$		$= 0.07$	Cg, PFC, NAcC, NAcSh, CPu, PO, BNST, HC		
Alcohol vs abstinence	$F_{1,406} = 0.48$		$= 0.50$			
Alcohol vs saccharine	$F_{1,406} = 1.44$		$= 0.25$			

Abbreviations used: BNST, bed nucleus of the stria terminalis; Ce, central amygdaloid nucleus; Cg, cingulate cortex; CPu, caudate putamen; GP, globus pallidus; HC, hippocampus; INS, insular cortex; IL, infralimbic cortex; LH, lateral hypothalamus; LS, lateral septum; M1, primary motor cortex; MS, medial septum; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; PAG, periaqueductal grey; PFC, prefrontal cortex; PO, preoptic area; Ra raphe nucleus; S1, primary somatosensory cortex; SN, substantia nigra; STh, subthalamic nucleus; TH, thalamus; VTA, ventral tegmental area

5.1.3. Behavioral effects of $MnCl_2$

The main drawback of using $MnCl_2$ in animal experiments is its neurotoxicity. Therefore, in order to ascertain that the 120 mg/kg/week $MnCl_2$ dose used in our experiments does not affect animals' behavior, fluid consumption and body weights were measured during the seven-day infusion period (Fig. 5.2). Generally, only minor effects of $MnCl_2$ on ingestive behavior and body weights were seen. Alcohol drinking was slightly reduced during the second and third infusion day ($F_{7,63} = 4.29$, $p < 0.001$), returning to the baseline by the fourth day. In contrast, water drinking was increased during the last infusion day in the water group ($F_{7,63} = 3.48$, $p < 0.01$), but neither the alcohol- nor saccharine-drinking rats exhibited significant changes ($F_{7,63} = 1.44$, $p = 0.22$ and $F_{7,63} = 1.64$, $p = 0.15$, respectively). Saccharine intake was also unaffected ($F_{7,63} = 0.86$, $p = 0.54$). $MnCl_2$ infusion decreased body weights significantly in all experimental groups (p 's < 0.001). The maximum body weight reduction, ranging from 7 to 7.6 %, was seen between third and fifth postsurgical days, after which the weights began to gradually increase.

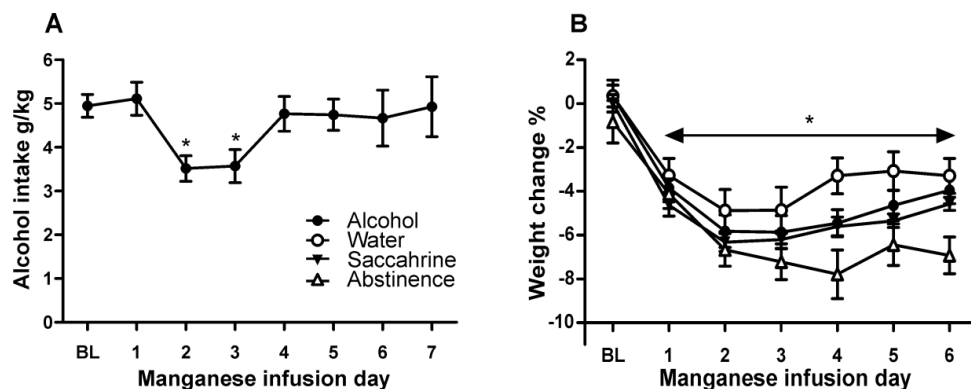


Figure 5.2 Effects of seven-day $MnCl_2$ infusion with osmotic minipumps on (A) alcohol consumption and (B) body weight of rats. Data are presented as mean (\pm SEM) for 8 subjects in each group. Weight differences are expressed as percentage change from the baseline (BL). * $p < 0.05$, significantly different from the BL defined as the mean of the previous seven days, Dunnett's test.

5.2. THE NUCLEUS ACCUMBENS CONNECTIONS IN THE REGULATION OF ALCOHOL DRINKING

5.2.1. Mapping of the nucleus accumbens connectivity with MEMRI

In order to assess the involvement of the NAc connectivity in the regulation of alcohol drinking and its modulation by the nonselective opioid antagonist NTX, we compared the MRI scans acquired immediately after intra-accumbal MnCl_2 injection with scans acquired 24 hours later (Fig. 5.3). The basis of this experiment is the ability of Mn^{2+} ions to use anterograde and retrograde transport to move along axons. Mn^{2+} ions can also be released to the synaptic cleft and taken up by postsynaptic neurons allowing activity-dependent tracing of neuronal tracts.

The within-group comparison of rats engaged in water and chronic alcohol drinking, as well as abstinent rats exhibited a similar rostral-caudal pattern of activation. In the caudal direction, this cluster included parts of the LH and ZI, extending to the SN and VTA. Alcohol-induced activation was also extended to forebrain regions, such as the INS, S1, secondary somatosensory cortex (S2), Amy, and PTg in the hindbrain. The within-group analysis of the connectivity between the NAc and various brain regions revealed nearly identical connection strengths in the water-drinking and abstinent rats. In the alcohol-drinking group however, long-term alcohol consumption significantly enhanced activation in most connections of the NAc. Naltrexone injections on the other hand, caused significant and consistent suppression of the connection strengths (Fig. 5.4). These data were also supported by between-group comparison, which revealed significantly higher activation in the alcohol-drinking group in comparison with control water in the INS, S1-2, M1-2, Amy, ZI, and PTg.

Alcohol-drinking rats receiving NTX injections displayed significantly decreased signal intensity in the neuronal tract seen in the other conditions. Between-subject comparison of alcohol-drinking rats treated with NTX and saline showed significantly lower activity in the orbital cortex (O), INS, CPu, and Amy in the NTX group. There was also a trend towards suppressed activity in the S1-2, M1-2, GP, VP, and ZI. Only two brain regions, namely, the S1-2 and PTg, exhibited activation in the NTX group compared to water.

Table 5.2 Experimental groups included in the mapping of the connectivity of the nucleus accumbens

Experimental group	N	Drinking history	Treatment
Water	9	Control rats that were drinking only water throughout the experiment.	None
Abstinence	9	Rats with a history of a voluntary alcohol drinking withdrawn from alcohol for 7 days before imaging	None
Alcohol	9	Rats that were voluntarily drinking 10 % alcohol throughout the experiment	Saline
Alcohol	10	Rats that were voluntarily drinking 10 % alcohol throughout the experiment	Naltrexone 3 mg/kg

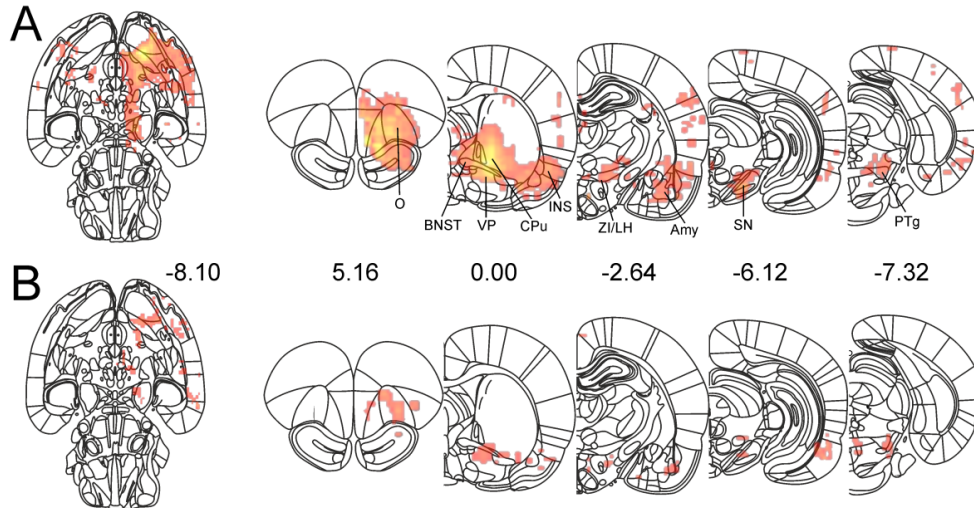


Figure 5.3 Differences in T_1 -weighted signal intensity between 1 hr and 24 hr after the $MnCl_2$ injections in alcohol (A) and naltrexone (B) group revealed by a voxel-wise paired t-test performed in SPM8. Activation heat maps were manually superimposed on corresponding sections from rat brain atlas (Paxinos and Watson, 2007). Numbers between the sections represent distance from bregma in stereotaxic coordinates. Abbreviations used: Amy, amygdala; BNST, bed nucleus of the stria terminalis; CPu, caudate putamen; INS, insular cortex; LH, lateral hypothalamus; PTg, pedunculopontine tegmental nucleus; SN, substantia nigra; VP, ventral pallidum; ZI, zona incerta.

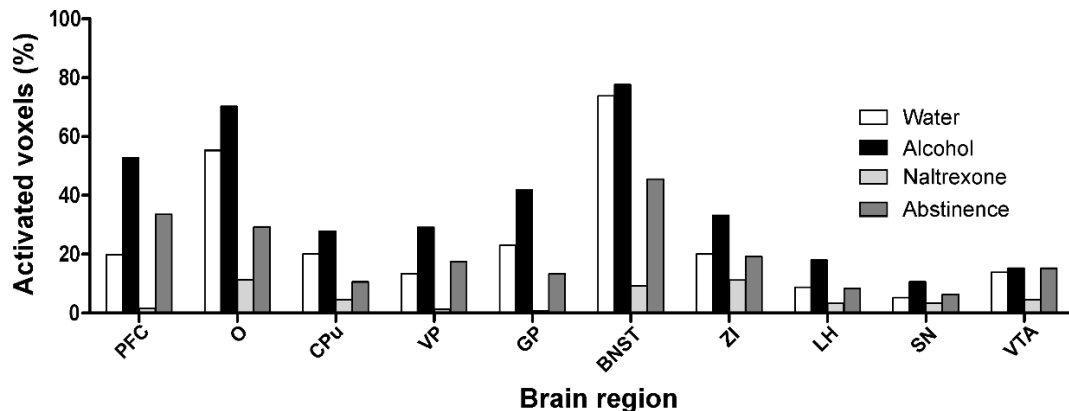


Figure 5.4 Strength of presumed input and output connections of the nucleus accumbens in different conditions presented as percentage of activated voxels of the whole ROI. The data were extracted from the group statistics using the REX tool. Abbreviations used; BNST, bed nucleus of the stria terminalis; CPu, caudate putamen; GP, globus pallidus; LH, lateral hypothalamus; PFC, prefrontal cortex, O, orbital cortex; SN, substantia nigra; VP, ventral pallidum; VTA, ventral tegmental area ZI, zona incerta.

5.3. THE ROLE OF CENTRAL LINEAR NUCLEUS IN PROPENSITY FOR AND REGULATION OF ALCOHOL DRINKING

5.3.1. Differences in basal brain activity between AA and Wistar rats

Statistical parametric mapping comparing alcohol-naïve AA and Wistar rats detected a generally lower basal functional activity in many brain regions in the AA rats. Both in the functional mapping and ROI analysis, the most significant difference between the AAs and Wistars was found in the CLi. In addition, the lower CLi activity was restored by chronic alcohol drinking, as shown by the analysis of the CLi mean T_1 -signal intensity in naïve and alcohol-drinking AA rats (Fig. 5.5 A). Based on these findings,

we hypothesized that the CLi might play an important role both in the innate alcohol preference and in the regulation of alcohol drinking. Therefore, in order to evaluate the involvement of CLi in alcohol drinking, we implanted guide cannulas into the CLi of AA rats and modulated its activity by intracranial injections of the GABA_A agonist muscimol and the GABA_A antagonist bicuculline.

5.3.2. Muscimol-induced increase in ethanol and saccharine consumption blocked by bicuculline coadministration

Results of one-way repeated measures ANOVA showed a dose-dependent increase in 10 % ethanol drinking in AA rats ($F_{3,27} = 3.17$, $p = 0.027$) after muscimol injections (Fig. 5.5). Further, Tukey's Multiple Comparison post-hoc tests revealed significant alterations after the 10 ng ($p < 0.01$) and 30 ng muscimol doses ($p < 0.001$). Moreover, ethanol consumption after the 10 ng and 30 ng muscimol was also significantly increased compared to the 3 ng dose ($p < 0.05$ and $p < 0.001$, respectively). No significant increase in ethanol consumption was detected after the 3 ng muscimol injections. Water consumption was not influenced by any muscimol dose ($F_{3,27} = 2.63$, $p = 0.30$). Co-injections of bicuculline with muscimol dose-dependently blocked muscimol-induced increase of ethanol consumption ($F_{2,23} = 3.88$, $p = 0.36$). Post-hoc Tukey's tests revealed that coadministration of the 10 ng muscimol with the 10 ng or 20 ng bicuculline doses failed to significantly increase ethanol consumption compared to the control group. However, only the 20 ng bicuculline produced a clear inhibitory effect. The 10 ng muscimol dose also significantly increased the consumption of 0.025 % saccharine solution (paired t-test, $p = 0.03$).

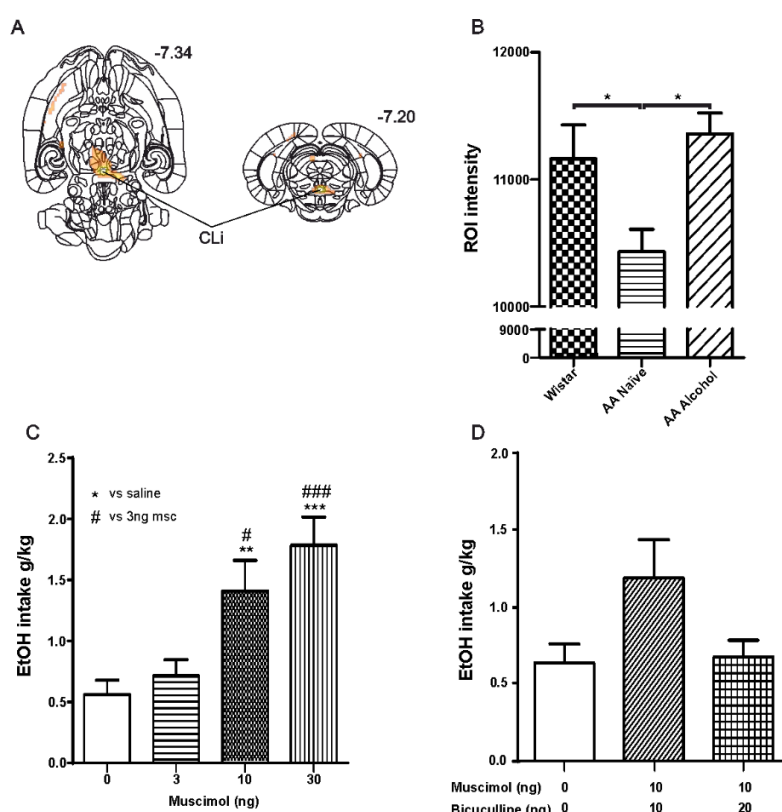


Figure 5.5 (A) Differences in brain activity revealed by T₁-weighted MRI signal intensity in naïve Wistar rats compared to naïve AA rats (Wistars > AAs). Statistical significance was thresholded at $p < 0.000001$ and the activation heat maps were manually superimposed on corresponding sections from the rat brain atlas (Paxinos and Watson, 2007). Numbers on the top right-hand corners of the slices indicate distance from bregma in stereotaxic coordinates. (B) Comparison of brain intensities extracted from the caudal linear nucleus (CLi) of naïve Wistar, naïve AA, and AA rats with alcohol drinking history. (C) Dose-dependent increase in 10 % alcohol consumption after intra-CLi muscimol injections. *, # $p < 0.05$; ** $p < 0.01$; ***, ### $p < 0.001$. Tukey's Multiple Comparison Test. (D) Blockade of the muscimol-induced increase in ethanol drinking by coinjections of bicuculline.

5.3.3. Intra-CLi muscimol does not affect locomotor activity of AA rats

Effects of the 10 ng intra-CLi muscimol injections on locomotor activity of AA animals were assessed with two-way repeated measures ANOVA with treatment as the between-subject factor and time points as the within-subject factor. The analysis revealed no significant effects of treatment ($F_{1,80} = 0.10$, $p = 0.76$), time interval ($F_{5,80} = 1.76$, $p = 0.13$) nor treatment x time interval interaction ($F_{5,80} = 0.64$, $p = 0.67$).

5.4. LONG-TERM EFFECTS OF MEPHEDRON AND METHAMPHETAMINE ON BRAIN ACTIVITY

5.4.1. Assessment of brain activity with MEMRI

Comparison of MRI scans of the drug-treated groups with saline controls revealed differential long-term effects of 4-MMC and METH on brain activity. Mephedron produced an increase in brain activity in the HT and several cortical regions, including the M1 and S1. METH, on the other hand, produced a widespread increase in brain activity in several brain regions including the Cg, INS, M1, striatum, TH, HC, Amy, SC, and Ra.

Further analysis of 22 regions of interest selected based on their potential involvement in effects of stimulant drugs showed that METH treatment produced significant decreases in brain activity in the NAc core, CPu, lateral GP, Cg, Ra, and SC. The decreases produced by METH ranged from 6 % to 7 % ($p < 0.05$). Conversely, treatment with 4-MMC produced a 10 % increase in brain activity and the effect was detected only in the HT ($p < 0.01$).

5.4.2. Influence of 4-MMC and METH on behavior

Unexpectedly, neither 4-MMC nor METH affected memory performance of rats. These data are not in accordance with previous studies, which reported significant decrease in memory performance after 4-MMC administration (Motbey et al., 2012b). In order to assess the development of locomotor sensitization, the differences in locomotor activity between day 1 and 4 of the treatment were calculated. A repeated measures ANOVA of locomotor activity between day 1 and 4 showed a significant main effect of treatment day ($F_{1,21} = 4.36$, $p < 0.05$), mainly produced by an increase on day 4. No significant treatment effect ($F_{2,21} = 1.96$, $p > 0.05$) or time x treatment interaction ($F_{3,27} = 0.90$, $p > 0.05$) was observed, indicating that sensitization did not take place.

6. DISCUSSION

In the first study we used MEMRI to demonstrate a widespread, region-specific increase in T_1 -weighted MR signal intensity after long-term voluntary alcohol drinking in many brain regions that have previously been shown to play a role in alcohol reinforcement. Moreover, saccharine drinking also increased signal intensity, mostly in the striatum. Persistently enhanced signal intensity was also detected in rats that abstained from alcohol drinking for one week.

To further investigate the involvement of the striatum in alcohol reinforcement, we also used MEMRI to characterize the activity of the NAc connections after chronic alcohol drinking and the modulation of alcohol-induced activity with the opioid antagonist NTX in the second study. The analysis revealed a clearly defined pathway originating from the NAc and projecting to the midbrain nuclei, including the VTA and SN. The activity of this pathway was clearly increased in the alcohol drinking rats compared to water drinking or abstinent rats. NTX was able to suppress the alcohol-induced activation of the striatum-midbrain tract.

In the third study, MEMRI was first used to assess differences in brain activity between naïve Wistar and alcohol-preferring AA rats. Further, based on the data showing a prominent reduction of brain activity in the CLi of the AA rats, we showed that voluntary alcohol drinking restored the low CLi activity and that intra-CLi injections of the GABA_A receptor agonist muscimol dose-dependently increased alcohol and saccharine drinking in AA rats without affecting water consumption. Moreover, we showed that the GABA_A antagonist bicuculline, coadministered with muscimol, blocked muscimol-induced increase in alcohol intake.

In the last study we used MEMRI for detecting drug-induced toxicity and showed that repeated high doses of METH resulted in long-lasting decreases of brain activity in several regions, including the striatum, GP, TH, HC, Ra, and a number of cortical areas. Conversely, 4-MMC produced an increase in brain activity in the HT and HC.

6.1. APPLICABILITY OF MEMRI

Various noninvasive *in vivo* imaging methods have been used in brain research, providing high resolution spatial images of anatomical or functional features of the brain at the time of scanning. When the use of the metal manganese as a contrast agent for imaging the brain was invented (Koretsky and Silva, 2004), it also opened up an opportunity to record both long-lasting and accumulated changes in brain activity. The manganese-enhanced magnetic resonance imaging, which was the main experimental method used in the present studies, is based on the paramagnetic abilities of the Mn^{2+} that can enter active brain cells through the voltage-gated Ca^{2+} channels and accumulate in them proportionally to their activity. Upon accumulation, Mn^{2+} causes a strong reduction of T_1 -weighted relaxation time of tissue water, which can be detected by MR imaging as increased signal (Aoki et al., 2004b; Lin and Koretsky, 1997; Natt et al., 2002).

Our own data demonstrates that the MEMRI method is sensitive enough for detecting neurophysiological changes in brain activity after alcohol and psychostimulant exposure. The pattern of METH-induced changes in brain activity observed in our studies (IV Fig. 4) largely overlaps with alterations observed in human METH users (Volkow et al., 2001). Furthermore, brain regions activated following long-term ethanol exposure in our alcohol-preferring AA rats (I Fig. 2) also overlap to a large extent with the regions reported to be activated using other experimental methods, such as metabolic mapping and measurement of c-Fos expression (Porrino et al., 1998; Yoshimoto et al., 2000). Together, these findings suggest that MEMRI is a reliable method for measuring brain activity alterations following long-term use of drugs, such as 4-MMC that are not easily detectable using other

immunological or neurochemical methods. In addition, MEMRI was also shown to be sensitive enough for uncovering changes in brain activity after various behavioral paradigms (Bangasser et al., 2013; Hoch et al., 2013), and our data from saccharine drinking demonstrates that also activation changes by rewarding substances with no pharmacological actions can be seen.

The main concern of using manganese in animal studies is its cardio- and neurotoxicity, and thus a critical issue for MEMRI imaging is to use as low MnCl_2 doses as possible that are, however, high enough to obtain the desired signal intensity. Chronic exposure to manganese leads to disorders resembling Parkinson's disease (Wolf and Baum, 1983). To circumvent the toxicity problem we chose to administer MnCl_2 from osmotic minipumps. Thereby animals do not receive an acute high dose of MnCl_2 , but instead it is released over one week, resulting in a total dose of 120 mg/kg/week. Little changes in ingestive behavior and a minor transient decrease in body weights observed in our animals suggested that the selected manganese dose combined with administration from osmotic minipumps produces only slight, if any, toxic effects (I Fig. 1). By showing that long-term voluntary alcohol drinking activates brain regions that have been previously linked to alcohol reinforcement (Noori et al., 2012), we demonstrated that MEMRI is a suitable method for imaging long-term changes in brain activity.

6.2. CHRONIC ALCOHOL INDUCES WIDESPREAD ALTERATIONS IN BRAIN ACTIVITY

The involvement of the nigrostriatal and mesolimbic DA systems in the reinforcing effects of alcohol and other drugs of abuse, as well as in the development of alcohol dependence is well documented (Koob et al., 1998). The influence of acute or chronic alcohol drinking on the brain has been tested using various imaging methods. For example, Porrino et al. (1998) used metabolic mapping to show that ethanol drinking induces changes in glucose utilization in brain regions belonging to the dopaminergic system (Porrino et al., 1998). Moreover, alcohol-consuming mice and rats exhibited increased c-Fos expression in a number of brain regions including the PFC, striatum, septum, BNST, Amy, several nuclei of TH and HT, SN, and VTA (Vilpoux et al., 2009). In our animals, long-term alcohol drinking activated major brain regions belonging to the dopaminergic pathways, including terminal fields of mesolimbic and nigrostriatal projections, such as the PFC, NAc, and CPu (I Fig. 2). Also human fMRI and PET studies reported activation of brain regions belonging to the mesolimbic system in subjects exposed to alcohol olfactory cues (Kareken et al., 2004), alcohol taste (Filbey et al., 2008), or alcohol challenge (Boileau et al., 2003) (see Table 6.1). Our functional activity mapping with MEMRI seems to be at least as sensitive as the previous approaches. In addition, agreement of findings obtained with MEMRI with human neuroimaging studies suggests that MEMRI could be a useful translational tool for assessing brain activity changes.

Table 6.1 Alcohol-induced brain activation. Examples of findings obtained with different imaging methods.

Method	Activated ROIs	Subjects and drinking	Reference
Metabolic mapping	medial prefrontal cortex nucleus accumbens lateral septum basolateral amygdala ventral tegmental area substantia nigra compacta	Male Wistar rats. Alcohol self-administration.	Porrino et al., (1998)
Functional MRI	nucleus accumbens ventral tegmental area	High risk drinkers. Alcoholic drink odors vs nonalcoholic odors.	Kareken et al., (2004)
Functional MRI	ventral striatum dorsal striatum ventral tegmental area substantia nigra medial prefrontal cortex olfactory cortex	Heavy drinkers. Alcohol taste vs taste or nonalcoholic drink.	Filbey et al., (2008)
PET	nucleus accumbens ventral caudate putamen	Nonalcoholic moderate drinkers. Consumption of alcohol in orange juice vs orange juice alone.	Boileau et al., (2003)

The widespread alcohol-induced brain activation recorded by MEMRI suggests that in addition to the dopaminergic pathways, long-term alcohol consumption probably recruits also other neurotransmitter systems. It is important to note that the alcohol-related alterations of brain activity exposed by MEMRI do not directly reveal the neurochemical basis of the observed activation patterns. However, based on knowledge of the anatomy of neurotransmitter systems, we can come up with suggestions for the involvement of some systems. Glutamatergic afferents to the VTA/SN originate from various regions between the PFC and the caudal part of the brainstem, including the BNST, lateral and medial HT, PO, PAG, SC, and mesencephalic reticular formation (Geisler et al., 2007). Activation of those glutamatergic afferent projections was shown to increase the firing rate of VTA DA neurons, indicating their importance in the action of drugs of abuse (Murase et al., 1993). Thus, the activation pattern in our rats is in agreement with the participation of glutamatergic pathways, which is also consistent with data showing high extracellular glutamate concentrations both in human alcoholics and alcohol-exposed rats in the VTA, striatum, and PFC (Ding et al., 2013; Hermann et al., 2012). GABAergic neurons most probably also contribute to the obtained activation pattern. The majority of the feedback projections from the striatum to the SN/VTA are GABAergic (Kalivas, 1993).

One might argue that brain activation revealed by MEMRI is merely caused by the direct pharmacological action of ethanol on the brain and does not represent activation of the brain reward mechanisms induced by alcohol. However, the remarkable similarity between alcohol-induced activation and the activation obtained following saccharine drinking (I Fig. 3), which does not presumably induce any pharmacological actions on the brain, proves that activation maps obtained with MEMRI at least partially represent activation of various neural processes involved in mediation of reward. High activation by saccharine drinking in the AA rats is also in accordance with previous reports describing high preference for sweetened solutions in alcoholic patients and rodents selected for high alcohol consumption (Kampov-Polevoy et al., 1997; Sinclair et al., 1992).

6.3. THE ROLE OF GABAERGIC SIGNALING IN THE CAUDAL VTA IN MEDIATING REINFORCING EFFECTS OF ALCOHOL

6.3.1. Influence of GABA_A ligands on alcohol drinking

The VTA has long been hypothesized to be of high importance in mediating the reinforcing properties of alcohol and other drugs of abuse. The VTA is populated mostly by the DAergic neurons, whose

activation after acute ethanol results in increased extracellular concentration of DA in the terminal fields in the ventral striatum (Di Chiara and Imperato, 1985). However, GABAergic (Olson and Nestler, 2007) and glutamatergic terminals (Hnasko et al., 2012) are also present in the VTA. It has been suggested that caudal and rostral parts of the VTA play differential roles in alcohol reinforcement. Moreover, we discovered that the naïve alcohol-preferring AA had lowered signal intensity in a brain region located in close proximity to the VTA, namely the CLi (III Fig. 1), and that intra-CLi injections of the GABA_A receptor agonist muscimol increased alcohol intake in AA rats (III Fig. 3 A), leading us to hypothesize that the GABAergic neurotransmission in the CLi is involved in regulation of alcohol drinking. Previous studies reported that microinjections of a GABA_A agonist to the caudal/posterior VTA increased the extracellular concentration of DA and its metabolites (Kalivas et al., 1990). Also, locomotor activity in animals that received microinjections of a GABA_A agonist to the caudal but not rostral VTA was increased (Arnt and Scheel-Kruger, 1979). However, the involvement of GABAergic signaling could be complicated by existence of two GABA_A mediated mechanisms in the VTA, as suggested by Ikemoto et al. (1998). This hypothesis is supported by previous studies that microinjections of a GABA_A agonist to the caudal VTA but not rostral VTA produces increased locomotor activity in rats, whereas increased locomotor activity was detected when a GABA_A antagonist was injected into the rostral but not caudal VTA (Arnt and Scheel-Kruger, 1979). In our experiment (III), muscimol injected to the CLi did not increase locomotor activity in alcohol-preferring rats, thus suggesting that CLi neurons are functionally distinct from those in the VTA. It is possible, however, that CLi neurons exert modulatory effects on VTA GABA neurons, hence affecting DA signaling in the VTA. Based on previous reports it is hypothesized that the GABA system located in the caudal VTA regulates the activity of the DA neurons. Therefore activation of GABA_A receptors with muscimol increases DA activity by inhibition of the GABA interneurons. The ones located in the rostral VTA on the other hand, control the GABA_A mediated tonic inhibition of the DA neurons, which is blocked by the GABA_A antagonists (Kalivas, 1993; Klitenick et al., 1992).

6.4. PERSISTENCE OF THE ALCOHOL-INDUCED CHANGES IN BRAIN ACTIVITY

As shown in I Fig. 2, chronic alcohol induces widespread changes in brain activity that could underlie several neuroadaptations associated with the gradual development of the compulsive forms of drug seeking (Hyman and Malenka, 2001). However, the brain regions and mechanisms underlying the development of those manifestations of addiction are not well known.

After seven days of abstinence following long-term alcohol exposure we observed remaining activation in some brain regions. The highest activity was seen in the NAc, CPu, PO, and BNST. Moreover, smaller activation clusters were also detected in the regions of the PFC including prelimbic and cingulate cortices, as well as in the ventral HC, and STh (I Fig. 2). Given that MnCl₂ was present only during the abstinence period, the observed activation patterns reflect adaptation produced by chronic alcohol exposure and perhaps brain processes involved in withdrawal. However, the interval between the last day of alcohol drinking and the beginning of MnCl₂ infusion was three full days. Symptoms of acute somatic withdrawal in rats usually subside between 24 to 48 hours after alcohol consumption (Zhu et al., 2013), and therefore they should not influence brain activity during the period from day 3 to day 10 after alcohol removal. It is more likely that the altered brain activity represents long-term and persistent adaptations accumulated during alcohol drinking. One of the known brain areas displaying such alterations in the PFC, where elevated glutamate levels persisted for at least 60 hours after withdrawal from chronic intermittent alcohol (Hermann et al., 2012). The mPFC is a key brain region involved in alcohol seeking in rats (Willcocks and McNally, 2013). Regions of the PFC have been implicated in drug seeking after cocaine withdrawal through the action on both D₁ and D₂ receptors (Peters et al., 2009). Moreover, inactivation of the prelimbic cortex was shown to attenuate alcohol

seeking and motivation (Willcocks and McNally, 2013). There are well-established prefrontal-accumbens glutamatergic connections (Sesack et al., 1989) that could contribute to increased activation in the NAc and possibly CPu. Differences in the c-Fos expression between alcohol withdrawn mice and controls were also present in the lateral septum, striatum, VP, and few cortical regions (Kozell et al., 2005). The STh has been demonstrated to be involved in motivation for natural rewards and drugs of abuse (Baunez et al., 2005). Moreover, lesions of this structure were shown to attenuate alcohol seeking and motivation (Lardeux and Baunez, 2008). The STh can be activated either by direct excitatory input from the prelimbic cortex or indirectly by a disinhibitory pathway originating in the PFC and passing through the NAc and VP (Maurice et al., 1998). Similar to the PFC, the ventral HC has also been linked with drug seeking (Lasseter et al., 2010).

6.5. IMPORTANCE OF THE ACCUMBAL CONNECTIVITY IN ALCOHOL ADDICTION AND INTERPLAY WITH THE OPIOIDERGIC SYSTEM

The endogenous opioid system is known to be involved in producing the reinforcing effects of alcohol, most probably by interactions with the mesolimbic DA system (Herz, 1997). The central hypothesis is that alcohol-induced release of β -endorphin in the VTA (Adams and Cicero, 1991) and subsequent disinhibition of the GABA interneurons (Johnson and North, 1992) results in DA release in the NAc (Di Chiara and Imperato, 1988). Either systemic or central administration of nonselective opioid antagonists, such as NTX, into the NAc or VTA decreased the accumbal DA release induced by alcohol (Gonzales and Weiss, 1998). Opioid antagonists have also been shown to reduce alcohol seeking and relapse, as well as to suppress alcohol self-administration (Hyytia and Kiianmaa, 2001).

6.5.1. Involvement of accumbal connectivity in functioning of a naïve brain

Under basal conditions, the comparison of T_1 -weighted scans obtained immediately after the intra-accumbal $MnCl_2$ injection with those obtained 24 hours later revealed a clear rostral-caudal pathway originating in the NAc and ending in the VTA and SN (**II** Fig. 2 A). Brain regions highlighted in this pathway included the VP, GP, and LH. These results are in accordance with previous findings obtained with retrograde tracing studies showing a wealth of connectivity between the NAc and VTA/SN (Watabe-Uchida et al., 2012), including the GABAergic feedback projections (Kalivas et al., 1993) or glutamatergic afferents from the BNST and LH to the VTA/SN (Geisler et al., 2007). Activation of the aforementioned brain regions was probably achieved by ability of Mn^{2+} ions to utilize anterograde axonal transport mediated by kinesin. However, we also showed activation in several cortical regions, including the mPFC, INS, and O. Given that Mn^{2+} is also at least partly transported retrogradely by dynein and retrograde kinesin (Matsuda et al., 2010), we hypothesize that glutamatergic afferents from the PFC to the striatum (Noori et al., 2012) are responsible for this activation.

6.5.2. Alcohol enhances the nucleus accumbens input and output connections

The enhancement observed in the alcohol group (**II** Fig. 2 C) was most probably a consequence of an alcohol-induced increase in the Mn^{2+} uptake by the accumbal medium spiny neurons expressing dopamine D_1 receptors. It has been previously shown that activation of those neurons causes inhibition of the VTA GABAergic neurons leading to increased DA release in the striatum (Kravitz et al., 2012). Stimulation of this pathway together with activation of strong excitatory inputs on DAergic neurons (Lobb et al., 2011) most likely contributed to the overall alcohol-induced enhancement of the rostral-caudal pathway showed by MEMRI. Accumbal connections with the regions of the PFC, which were activated under the basal conditions, also displayed enhanced signal intensity after alcohol drinking. As

mentioned before, there are well established glutamatergic connections between the PFC and NAc (Noori et al., 2012). Moreover, prefrontal regions, such as INS, have been shown to be activated by alcohol and alcohol related cues (Claus et al., 2011). Therefore, we hypothesize that alcohol-induced enhancement of these connections results from retrograde Mn^{2+} transport.

6.5.3. Opioid antagonist naltrexone blocks alcohol-induced activity of accumbal connections

Inhibition of alcohol-induced brain activation by systemically given NTX (**II** Fig. 2 D) was most probably produced by a concerted effect from blockade of μ -opioid receptors in various brain regions. Many studies have reported effects of centrally administered NTX on alcohol-induced activation of transmitter systems. For example, intra-VTA NTX was shown to block alcohol-induced disinhibition of DAergic neurons and thus prevent DA release in the NAc (Valenta et al., 2013). Moreover, blockade of opioid receptors directly in the NAc with NTX or the δ -opioid receptor antagonist naltrindole attenuated alcohol-induced DA release in the NAc (Acquas et al., 1993). NTX injections into another important brain region, the Amy, were able to reduce alcohol reinforcement in rats (Heyser et al., 1999).

6.1. EFFECTS OF METH AND 4-MMC ON BRAIN FUNCTION

Stimulants, such as METH are known to primarily target brain regions that are dependent on dopaminergic and serotonergic signaling (Howard et al., 2013). Long-term exposure to high doses of METH causes various neurotoxic effects, such as degeneration of DA and 5-HT neurons followed by cell death, reduced concentration of DA and 5-HT, as well as their transporters and enzymes required for their synthesis (Schroder et al., 2003). We believe that the observed decrease in brain activity in several brain regions, including striatum and Ra after METH treatment (**IV** Fig. 4) arises from METH-induced degeneration of dopaminergic and serotonergic terminals, which are unable to take up Mn^{2+} ions from the intracellular space, thus producing less signal than the control condition. These findings are in line with previous animal and human studies describing brain regions vulnerable to METH-induced neurotoxicity, as well as those showing altered brain metabolism (Schroder et al., 2003; Volkow et al., 2001). Conversely to METH, 4-MMC produced increased brain activity in several brain regions. However, these effects were limited to only a few brain regions and were considerably smaller than those produced by METH. This is rather surprising considering that METH and 4-MMC exert similar effects on monoaminergic neurotransmission by blocking their reuptake and enhancing their release (Baumann et al., 2012). Recent studies, however, have demonstrated that 4-MMC is ineffective in various neurochemical or immunological tests of neurotoxicity (Baumann et al., 2012), which may explain its limited effects compared to METH.

6.2. TRANSLATION VALUE AND CHALLENGES OF MEMRI

Our experiments demonstrated that MEMRI is a versatile experimental method for detecting global long-term alterations in brain activity after pharmacological or behavioral intervention, as well as for tracking changes in selected brain circuits. The benefits of MEMRI include the ability to assess functional brain activation in the absence of anesthesia, which is based on the retention of Mn^{2+} ions in neural structures following activity-dependent uptake. Furthermore, MEMRI is independent of the nonlinearity caused by neurovascular coupling associated with fMRI BOLD contrast, and the spatial resolution offered by MEMRI is reasonably good. As a downside, the temporal resolution of MEMRI is rather poor compared to BOLD fMRI, which makes it difficult to discern the specific temporal association of the observed activity changes with the preceding challenges. Therefore, it is possible that the MEMRI signal is a summation of many activations that are not directly associated with the primary

challenge, but are counter adaptations, e.g., by autonomic regulation. On the other hand, the accumulated activity changes can also reveal interesting adaptive processes, as seen here during early abstinence from alcohol drinking.

Considering the harmfulness of the main component of MEMRI, namely manganese, a special attention has to be given to balancing the doses of MnCl_2 , because manganese-induced disruption of the physiological homeostasis may itself cause brain disorders that could greatly interfere with outcomes of the experiments. Manganese-induced toxicity can be reduced by the use of osmotic minipumps for MnCl_2 delivery, as described here. In addition, more work has to be done towards understanding the dynamics of Mn^{2+} in the body, including the proportional uptake in neurons and glial cells, and other issues related to pharmacokinetics, which might maximize the availability and uptake rate of Mn^{2+} into the brain, thus allowing the use of lower MnCl_2 concentrations.

Comparison of animal studies of any sort with corresponding human studies always raises doubts regarding the translational value of animal models and their applicability to human conditions. Imaging is not an exception, and therefore great care must be exerted in comparing the imaging data from MEMRI with those obtained from fMRI in humans. However, it is intriguing that parallel findings from rodent and human imaging data are emerging, such as the identification of the ventral striatum and orbitofrontal cortex as important sites for naltrexone's action (Myrick et al., 2008; Schacht et al., 2013b).

These data speak for the validity of investigating drug-induced alterations in brain functioning in rodent models, and, furthermore, suggests that preclinical imaging including MEMRI can be used as a translational tool that might help to reduce the gap between animal studies and clinical investigations.

7. CONCLUSIONS

- Ia. Long-term voluntary alcohol drinking produces a region-specific increase in brain activity in many cortical and subcortical regions that have been previously linked with alcohol reinforcement, including the ascending dopamine systems and their afferents. Moreover, a similar pattern of activation after long-term saccharine drinking further indicates that these data represent changes in brain reward circuitry, and point to commonalities between alcohol and saccharine drinking
- Ib. Persistent brain activity following the 7-day abstinence from alcohol drinking suggests development of alcohol-induced brain adaptations, which may contribute to alcohol seeking behavior.
- II. Increased activation of the accumbal neural connections during alcohol consumption and suppression of alcohol-induced activation by naltrexone suggests that the nucleus accumbens plays an important role in the regulation of the alcohol drinking and its attenuation by opioid antagonists
- III. Decreased basal activity of the CLi in the AA rats and the reversal thereof by alcohol suggests that the CLi is one of the key brain nuclei mediating the propensity for high alcohol drinking. Increased alcohol intake after intra-CLi injections of the GABA_A agonist muscimol suggests the involvement of GABAergic neurotransmission in CLi's role in alcohol reward.
- IV. Methamphetamine produces long-term widespread decreases in activation in brain regions that are heavily dependent on monoaminergic signalling. Opposite and significantly weaker pattern of long-term changes obtained after mephedron use suggest lower severity of toxic effects produced by the mephedron compared to methamphetamine.

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